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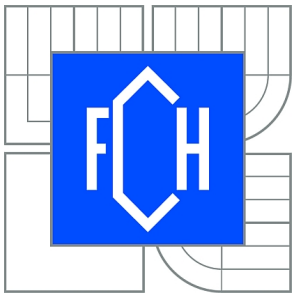

FAKULTA CHEMICKÁ
ÚSTAV CHEMIE MATERIÁLŮ

FACULTY OF CHEMISTRY
INSTITUTE OF MATERIALS SCIENCE

SEPARACE A CHARAKTERIZACE ATELOKOLAGENU

ATELOCOLLAGEN SEPARATION AND CHARACTERIZATION

BAKALÁŘSKÁ PRÁCE
BACHELOR'S THESIS

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BRNO 2014

Vysoké učení technické v Brně
Fakulta chemická
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Zadání bakalářské práce

Číslo bakalářské práce:	**FCH-BAK0760/2013**	Akademický rok: **2013/2014**
Ústav:	Ústav chemie materiálů	
Student(ka):	**Zuzana Konečná**	
Studijní program:	Chemie a chemické technologie (B2801)	
Studijní obor:	Chemie, technologie a vlastnosti materiálů (2808R016)	
Vedoucí práce	**Ing. Lucy Vojtová, Ph.D.**	
Konzultanti:		

Název bakalářské práce:

Separace a charakterizace atelokolagenu

Zadání bakalářské práce:

1) Vypracování literární rešerše týkající se vlastností atelokolagenu a jeho separace z kolagenové hmoty.
2) Na základě literární rešerše navržení postupu separace rozpustného atelokolagenu z komerčního bovinního kolagenu.
3) Experimentální práce dle navrženého postupu.
4) Optimalizace podmínek izolace atelokolagenu s cílem získání maximální konverze a maximální čistoty.
5) Charakterizace vlastností získaného atelokolagenu.
6) Závěr

Termín odevzdání bakalářské práce: 23.5.2014

Bakalářská práce se odevzdává v děkanem stanoveném počtu exemplářů na sekretariát ústavu a v elektronické formě vedoucímu bakalářské práce. Toto zadání je přílohou bakalářské práce.

Zuzana Konečná Student(ka)	Ing. Lucy Vojtová, Ph.D. Vedoucí práce	prof. RNDr. Josef Jančář, CSc. Ředitel ústavu

V Brně, dne 31.1.2014

prof. Ing. Jaromír Havlica, DrSc.
Děkan fakulty

ABSTRAKT:

Cílem předložené bakalářské práce byla optimalizace separace rozpustného atelokolagenu z komerčně dostupného hovězího kolagenu a jeho následná charakterizace. Kollagen byl podroben enzymatickému trávení pomocí pepsinu a následně přečištěn pomocí filtrace, centrifugace a dialýzy. Získané vzorky byly následně charakterizovány pomocí infračervené spektrometrie, elektroforézy v polyakrylamidovém gelu a Hartree-Lowryho metody pro stanovení celkového obsahu proteinů.

Vhodný časový rozsah enzymatického trávení pro získání vyhovujících výtěžků, byl určen na 60 – 70 hodin při hmotnostním poměru pepsinu a kolagenu 1:25. Nejvyšší výtěžek (72.6 %) byl získán při tomto poměru a době reakce 66 hodin. Dostatečné filtrace, která byla pro čistotu produktu klíčová, byla zajištěna kombinací filtračních technik s následnou centrifugací. Kvalita produktů z hlediska molekulových hmotností byla charakterizována pomocí gelové elektroforézy. Rozpustnost a její závislost na pH a koncentraci NaCl v roztoku byla měřena pomocí Hartree – Lowryho metody. Poměr mezi helikálním kolagenem a denaturovaným kolagenem ve vzorku byl měřen pomocí infračervené spektroskopie. Výsledná spektra se však nejevila zcela věrohodná. Jako vhodnější metodu pro zkoumání struktury kolagenu je možné použít například metodu cirkulárního dichroismu.

ABSTRACT:

The aim of the presented bachelor thesis was to optimize separation of soluble atelocollagen out of commercially available bovine collagen and its characterization. Collagen went through an enzymatic digestion using pepsin as a protease and subsequently purified by filtration, centrifugation and dialysis. Obtained samples were characterized using infrared spectrometry, polyacrylamide gel electrophoresis and Hartree-Lowry assay for total protein determination.

Suitable digestion time range to gain sufficient yields was decided from 60 – 70 hours at pepsin/collagen weight ratio 1:25. Highest yield (72.6 %) was obtained at mentioned ratio after 66 hours of reaction. Sufficient filtration, which was decisive for final product purity, was assured by combination of several filtration techniques with centrifugation. Quality of prepared product according to their molecular weight was characterized by gel electrophoresis. Solubility and its dependence on pH and NaCl concentration in solution were measured by Hartree – Lowry assay. Ratio between helical collagen and denaturated collagen was measured by infrared spectroscopy. Obtained spectra yet did not seem fully reliable and provided only a rough estimate. More accurate information about structure could be achieved by using circular dichroism spectroscopy.

KLÍČOVÁ SLOVA:

atelokolagen, kolagen, pepsin, enzymatické štěpení

KEYWORDS:

atelocollagen, collagen, pepsin, enzymatic digestion

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PROHLÁŠENÍ:

Prohlašuji, že jsem bakalářskou práci vypracovala samostatně a že všechny použité literární zdroje jsem správně a úplně citovala. Bakalářská práce je z hlediska obsahu majetkem Fakulty chemické VUT v Brně a může být využita ke komerčním účelům jen se souhlasem vedoucího bakalářské práce a děkana FCH VUT.

.....

podpis autora

DECLARATION:

I declare that my bachelor thesis was worked up independently and that used references are quoted correctly and fully. The content of above mentioned thesis is considered a property of BUT Faculty of chemistry and can be used for commercial purposes only with the supervisor's and dean's consents.

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author's autograph

PODĚKOVÁNÍ:

Touto cestou bych ráda poděkovala vedoucí práce Ing. Lucy Vojtové, Ph.D. za důležité rady a odborné vedení. Ráda bych také poděkovala Ing. Lukáši Zubalovi za spolupráci v laboratoři a všem kolegům z laboratoře za přátelské prostředí a pomoc při práci. Dále bych chtěla poděkovat Ing. Petře Matouškové a kolektivu z laboratoří biochemie za ochotné rady a poskytnutou možnost měření u nich v laboratoři a Ing. Janu Žídkovi, Ph.D. za pomoc při zpracování grafických dat. Na závěr bych chtěla poděkovat rodině a všem přátelům za podporu.

CONTENT

1 INTRODUCTION

Collagen is the most abundant fibrous protein in human as well as in all the mammalian body. It can be found all over the body, mainly as a component of connective tissue. Its main function is to keep the tissue compact, mechanically tough and flexible.

As a naturally produced material, collagen has very low antigenicity and good biodegradability. That is why it is very often used in medical application, such as tissue engineering, cardiovascular surgery, dermatology, orthopaedics and many others. It is also widely used in pharmacy, food and cosmetic industry.

Atelocollagen is a collagen without terminal peptide groups. It can be obtained by protease digestion of collagen molecules and is considered to have even smaller antigenicity. Using this soluble form of collagen is better at observing its self-arrangement and available to be easily modified by other natural or synthetic polymers.

However, successful separation of atelocollagen is not that simple, as it depends on many procedural conditions and variable settings as well as on chosen material.

2 THEORETICAL PART

2.1 Collagen

Collagens are the most widely spread fibrous proteins in mammalian body. It forms about 30 % of whole body protein content. Main role of these fibrils is to maintain tissue compactness and provide mechanical and structural properties that each tissue demands[1].

2.1.1 Structure of collagen

Collagen is a protein, synthesized of 20 commonly occurring amino acids. However, it is very unique as for its amino acid sequence pattern, high grade of posttranslational modification and specific intermolecular cross-links[1].

Collagen occurs at many structural levels (Figure 1). Primary structure consists of linear amino acid sequence. Individual acids hold together by peptide bonds. These tough, planar bonds combine amino group of one amino acid with carboxyl group of another one. Amino acid composition indicates infrequently high content of Gly, nearly 33 %. It means that almost every third amino acid is Gly and refers to repeating structural pattern $(Gly\text{-}X\text{-}Y)_n$. Primary structure forms a more complex typical α-helices or β-plated sheets, as the most stable of all possible secondary structures. Secondary structures are connected to make a three-dimensional tertiary structure – triple helix. This structural level is common for all the collagen types. The collagen triple helix consists of three left-handed polypeptide chains, simultaneous and alternating in right-handed sense around theirs common axis. The three chains are connected by an inter chain hydrogen bond. Tertiary structures then form three dimensional quaternary structure of a multi-unit protein[1, 2].

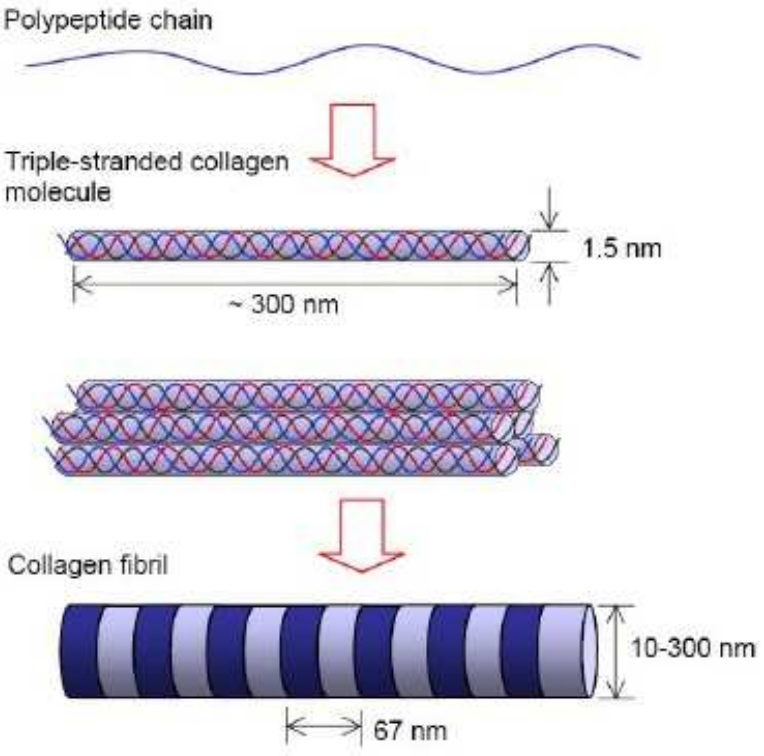

Figure 1: Structural levels of collagen[3]

2.1.2 Mechanical properties

Collagen fibre shows a characteristic shape of the stress-strain curve (Figure 2). The nonlinear parts are caused by natural form of collagen without any external force impact. By a "toe region" it is meant the lower area of a curve in which collagen tissue normally works. The tangent modulus of collagen is placed between the values 350 and 1000 MPa, reported tensile strength is between 50 and 100 MPa. Stability dependence of collagen on temperature and pH value is used by biomaterial scientist as variables for digestion and extraction process[2].

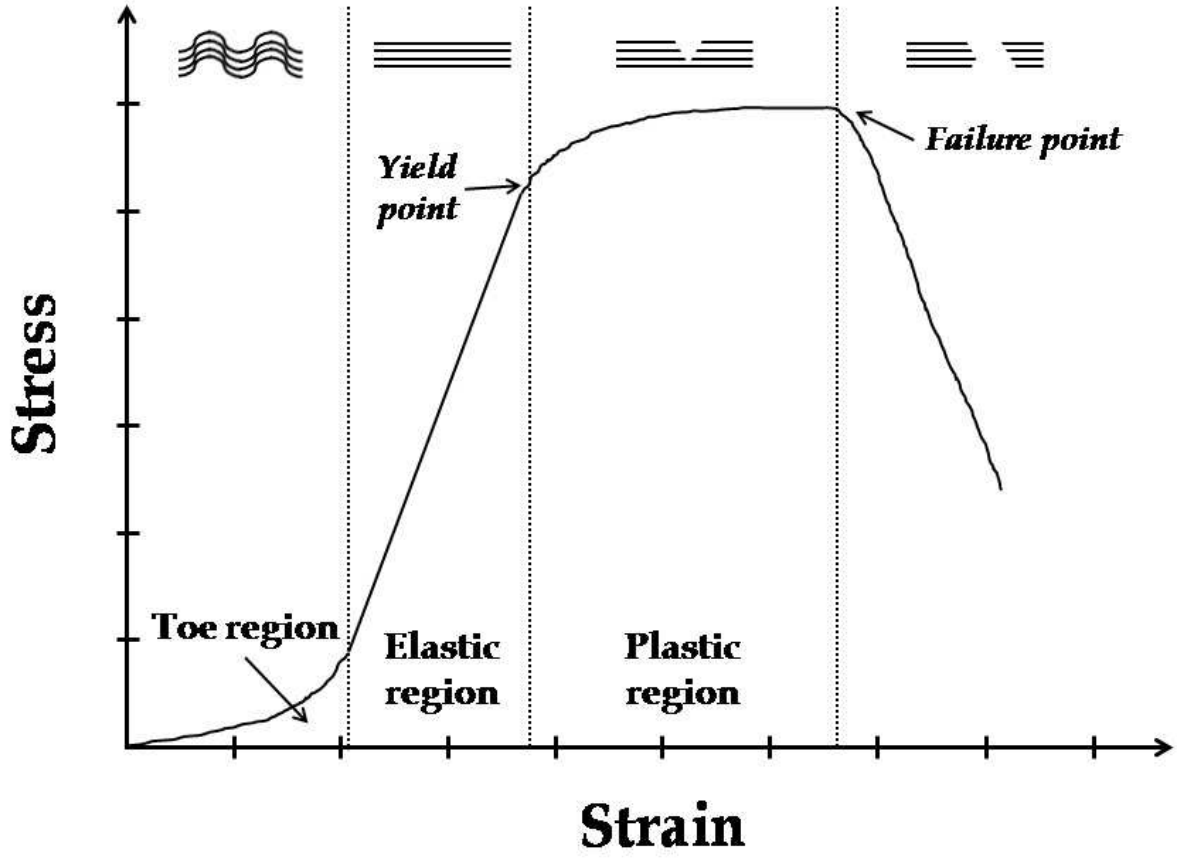

Figure 2: Characteristic stress-strain curve of collagen[4]

2.1.3 Occurrence and function

Currently, there are 28 types of collagen to be found in almost every kind of human or animal body tissues. Specific distribution of sites known 21 types is shown in Table 1. Each collagen family occurs in characteristic tissue type, has a specific structure with a triple-helix domain length and carries out various functions. On that basis, collagen can either form fibrils, anchored fibrils, fibril surface or membranes. The most frequent fibril-forming types of collagen are type I, II, III, V and XI. Type I, covering up to 90 % of all body collagen, is mostly found in bones, tendon, cornea and ligament. Type II forms fibrils in cartilage, vitreous and notochord. Type III collagen usually accompanies the type I in the extensive tissue filaments. Types V and XI are present in fibrils along with types I, II and III. The non-

fibrillar collagens, such as type IV, VIII, X and more, are not so plentiful in total, yet in specific tissue locations they can be prevalent[1,2].

Table 1: Distribution of different types of collagen along with their α-chain composition[1]

Type	α-Chains	Distribution
I	α1, α2	Bone, tendon, ligament, cornea, dermis
II	α1	Hyaline cartilage
III	α1	Skin, vessel wall, distensible tissues
IV	α1-α6	Basement membrane
V	α1-α3	Same as Type I, but smaller contribution
VI	α1-α3	Dermis, cartilage, placenta, lungs, inter vertebral disc
VII	α1	Dermal-epidermal junctions, cervix, oral mucosa
VIII	α1, α2	Endothelial cells, Descemet´s membrane
IX	α1-α3	Cartilage, cornea
X	α3	Foetal or juvenile cartilage
XI	α1-α3	Cartilage
XII	α1	Ligament, tendon, perichondrium
XIII	α1	Epidermis, hair follicle, intestine, liver, lung
XIV	α1	Dermis, tendon, vessel wall, placenta, lung
XV	α1	Fibroblasts, smooth muscle cells, kidney, pancreas
XVI	α1	Fibroblasts, keratinocytes, amnion
XVII	α1	Dermal-epidermal junctions
XVIII	α1	Lung, liver
XIX	α1	Rhabdomyosarcoma
XX	α1	Embryonic skin, 10terna cartilage, tendon
XXI	α1	Vessel wall

2.1.4 Biosynthesis and biochemistry

Collagens are planned for extracellular matrix and their biosynthetic process takes place in the endoplasmic reticulum. Biosynthesis is subsequently followed by transportation trough the Golgi and secretion from the cell. Whole process takes about 15 minutes (type I). During the biosynthetic process collagen reacts with different types of enzymes that enable various reactions[1].

Collagen fibrils and other forms are highly stable structures with lifetime at least six months, usually much more. Time of degradation usually differs by a specific collagen type. However, a controlled degradation of collagen can be desirable, as it is used in wound healing and tissue engineering[1].

2.1.5 Application

All together with gelatine, collagen is most used protein of all. Application covers natural and synthetic leather, various cosmetic and food industry products and nutrition supplements.

Another important field of use is medicine (Table 2). Because collagen can be found in all kind of body tissue, it is often used as biomaterial for implants and tissue engineering[1].

Table 2: Examples of the applications of collagen and gelatine products in medicine

Medical area	**Application**
Cardiovascular surgery	Vessel replacement, heart valves
Dentistry	Periodontal attachment, alveolar ridge augmentation
Dermatology	Tissue augmentation
Dressings	Wound repair, burn treatment, graft site treatment
Esophageal surgery	Augmentation
General surgery	Hernia repair, adhesion barriers, hemostasis, adhesives
Neurosurgery	Nerve conduits, nerve repair
Ophthalmology	Corneal graft, vitreous replacement, retinal reattachment
Orthopaedics	Bone repair, cartilage reconstruction, ligament repair
Otology	Tympanic membrane replacement
Urology	Ureter replacement, renal repair, urinary incontinence

2.2 Atelocollagen

Atelocollagen is a term for collagen, processed by enzymatic digestion. During the digestion, a telopeptides are removed from the collagen triple helix and bonds between each of the triple helix, forming the collagen molecule, undergo a cleavage (Figure 3). By undergoing this process, the solubility of a final product increased, which is mainly demanded in biomedical applications.

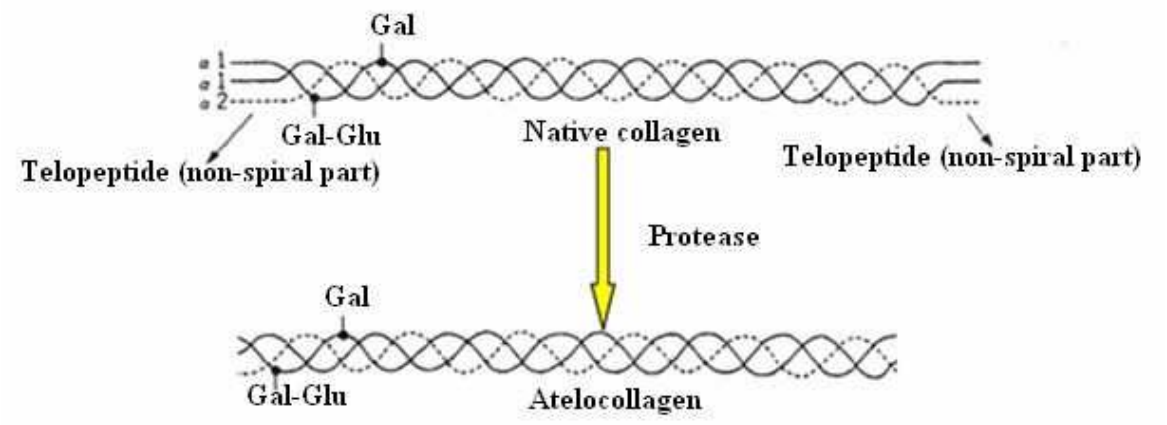

Figure 3: Atelocollagen molecule[5]

2.2.1 Separation process

Separation process itself not only includes the enzymatic digestion, but also some other steps that precede or follow it. There are many variables in each of the step, which differ according to used source and type of tissue and a collagen type it contains. Whole separation

process could be run at different temperatures, usually at 4 °C (in fridge) or at room temperature.

2.2.1.1 Animal and tissue choice

Preparation starts with extraction of a collagen from the tissue. The choice of an animal as a tissue source usually depends on its availability in the region. Mostly used is fish[6-10; 12-16], bovine[11] and pork tissue, to a lesser extent rats or other animals. Type of the tissue, such as skin, bone and other, is than selected according to a collagen type needed (Table 1).

2.2.1.2 Preparation of collagen sample

Separation starts with removing the desirable tissue from the residue and cutting it into small pieces to increase the effect of subsequent extraction steps. Soft tissue is usually cut with scissors or knife and solid tissue such as bones needs to be broken by a hammer[6-16].

Extraction of non-collagenous proteins

Unwanted proteins in a tissue pieces are then removed using mainly alkaline extraction by NaOH, usually at concentration of 0.1 M and sample/alkaline solution ratio from 1:5 to 1:30[6-10; 12-16].Time of the extraction ranges from 6 to 48 hours and the alkaline solution is changed on a regular basis. After removing the supernatant, samples are washed in distilled water to reach the neutral pH[6-16].

Decalcification

If we choose to use bones as a raw material, decalcification step needs to be involved to the separation process. For this purpose, 0.5 M EDTA or EDTA-2Na is usually used at sample/EDTA ratio 1:10. This step is very time consuming and lasts usually around 5 days[7; 10; 16].

Fat remove

Tissue is than defatted using either diethyl ether[6], 10 % butyl alcohol with solvent/solution ratio 1:10[7; 12, 14-16] or 1:20[8] or commercial detergent[10; 13]. Time of a fat extraction ranges between 24 and 48 hours. After that, tissue is again washed with distilled water[6-10; 12-16].

2.2.1.3 Protease digestion

Digestion usually runs in 0.5 M acetic acid and pH value of the solution range from 2 to 4[6-12]. pH is modified in the view of the fact that each enzyme is active in specific pH scale. Mostly used enzyme is pepsin from porcine stomach mucosa. Sample/solution ratio has main impact on viscosity and usually range from 1:10 to 1:100[7-9; 11-16]. Time of digestion differs according to pepsin concentration and reaction temperature and range between 24 and 72 hours[6-9; 11-16]. Pepsin /sample ratio usually range from 1:10 to 1:100[6-16].

Pepsin is one of the digestive enzymes found in animal stomach and is secreted as pepsinogen. In acidic environment, pepsinogen immediately turns into pepsin (Figure 4). Pepsin activity is declared to be from 1.5 to 3.5 pH[18].

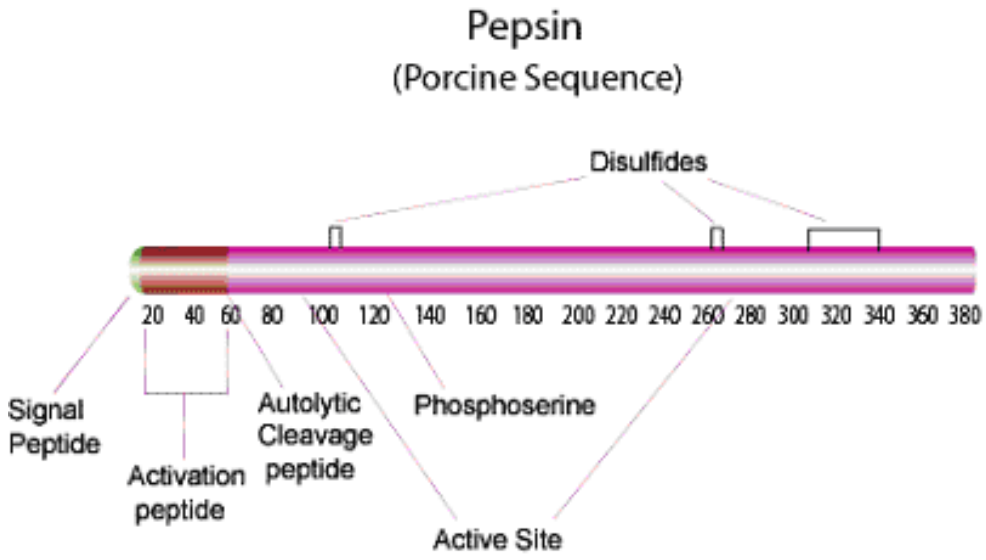

Figure 4: Pepsin sequence arrangement[14]

2.2.1.4 Purification

Filtration

After desired time, the mixture is filtrated or centrifuged to obtain a clear solution. There are many types of filtration devices available and choosing the right type or a combination of filtration techniques can be decisive for obtaining an unpolluted product. Collagen solution can be filtrated trough filter paper[6], cheesecloth[8; 10; 12; 14-15], frit, metal filter or various filtration devices.

Another possibility is to replace the filtration with centrifugation[7; 9-13; 15]. Advantage of this way to obtain the clear solution is its variable settings and good speed, in comparison with classical filtration techniques.

Insoluble residue after filtration could be processed again to increase the yield as desirable.

Precipitation

Reaction is then stopped by increasing the pH to alkaline area from pH 7 to 8. For this purpose sodium hydroxide, tris or phosphate buffered saline can be used. Collagen is then precipitated by adding NaCl solution to a final concentration from 0.7 to 3 M. Precipitated solution can be left over night to observe its stability[9]. Precipitate is then collected by centrifugation. Whole salting out procedure can be repeated once or two times again to increase the yield, because even after precipitation and centrifugation, there is still some collagen left in the solution[6-16].

Centrifugation

Centrifugation is a type of sedimentation, which uses centrifugal force to speed up the process. Different acceleration, time duration and temperature can be set at commonly used centrifuge (Table 3)[6-16].

Table 3: Variable centrifugation settings used in articles

Article	Acceleration [g]	Duration [min]
6	20 000	40
7	12 500	40
8	15 000	30
9	10 000	20
10	20 000	30
11	22 000	15
12	20 000	60
13	9 000	20; 30
14	20 000	60
15	10 000	15
16	15 000	60

Dialysis

Dialysis is a process, used for removing low molecular weight unwanted components from the polymer solution (Figure 5). After centrifugation, collagen sample is dissolved in 0.5 M acetic acid and dialysed against 0.05-0.1 M acetic acid, distilled water or both, respectively, to remove salts. Time of dialysis is usually 3 days and more with periodical change of solution[6-16].

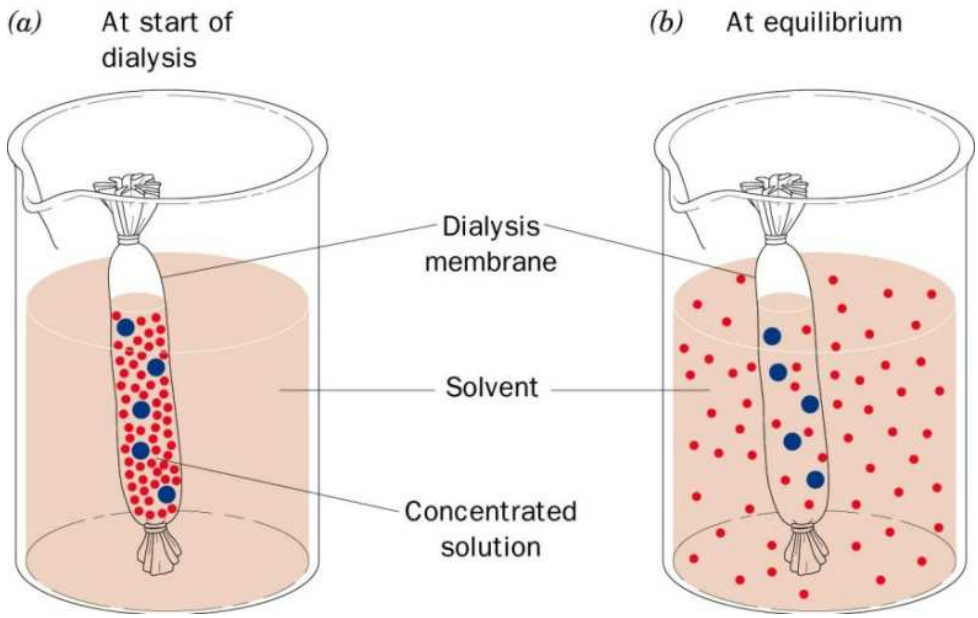

Figure 5: Principle of dialysis[22]

Lyophilization

After dialysis, collagen solution is frozen and lyophilized using a freeze dryer[6-16]. General scheme of freeze dryer is shown below (Figure 6).

Lyophilization is a dehydration method using a low pressure to dispose of all the water content in frozen sample. Low pressure allows the water to sublime straight from solid phase to gas phase[21]. After lyophilisation, collagen sample is in solid dry state, ready to be characterized.

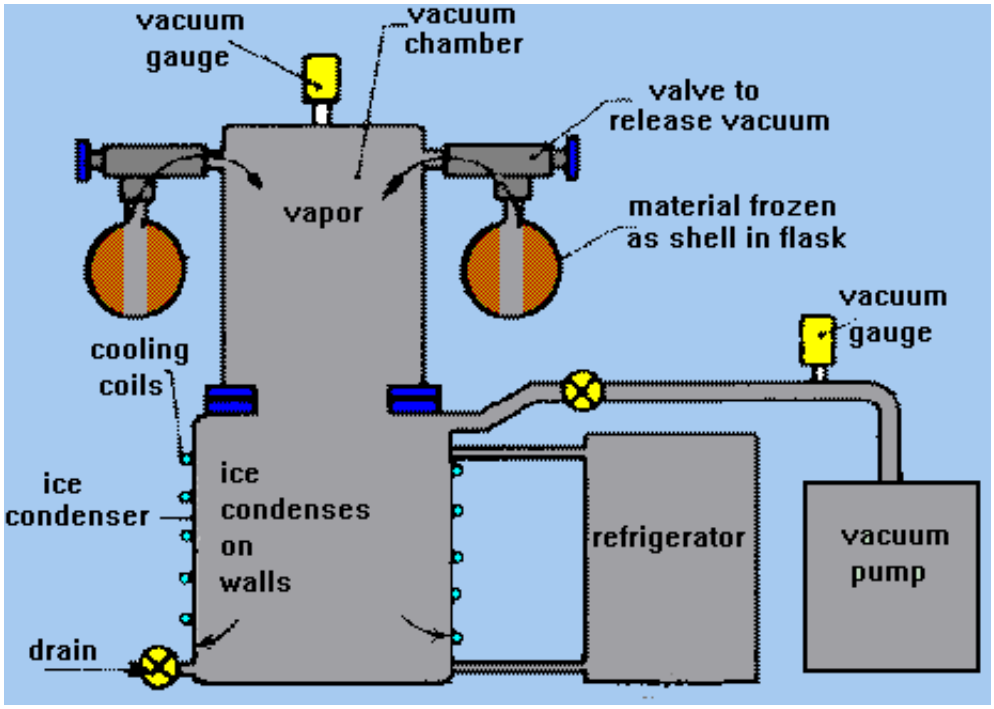

Figure 6: Scheme of a freeze – dryer[23]

2.2.2 Methods of characterization

2.2.2.1 Electrophoretic methods

Electrophoresis describes a migration of charged particles in an electric field. Diverse components migrate with different speeds and form separate zones. The electrophoretic mobility depends on charge, size and shape of the molecule, together with viscosity, pore size, buffer pH, ionic strength and temperature of the medium. Electrophoretic migration is characterized as:

$$v = \mu \cdot E$$

v …migration speed

μ …electrophoretic mobility

E …electric field strength

Electrophoretic mobility is characterized as:

$$\mu = \frac{q}{6 \cdot \pi \cdot \eta \cdot r}$$

q …charge of the particle

η …solution viscosity

r …radius of the particle

From the above mentioned equation is apparent that small particles with higher charge have higher mobility and large particles with lower charge have lower mobility. Effective electrophoretic mobility is the one we measure during the electrophoresis and is usually lower than theoretical, as it depends on pH and used buffer. Different particles are successfully separated only if their effective electrophoretic mobilities differs enough[19; 20].

Polyacrylamide gel electrophoresis (PAGE) with addition of sodium dodecyl sulphate (PAGE-SDS)

Polyacrylamide gel is inert, transparent and mechanically tough and can be prepared by polymerization of acrylamide monomer (AA) using N,N´-methylenebisacrylamide (BIS) as cross-linking agent, ammonium persulfate (APS) as initiator and N,N,N´,N´-tetramethylenediamine (TEMED) as stabilizing agent for free-radicals. Oxygen has an inhibitive effect on the polymerization and can be removed by ultra-sonification.

Properties of the gel are mainly affected by its porosity and degree of cross-linking, meaning by ratio between AA and BIS, which usually range from 100:1 to 20:1. Total concentration of AA is usually from 3 to 15 %.

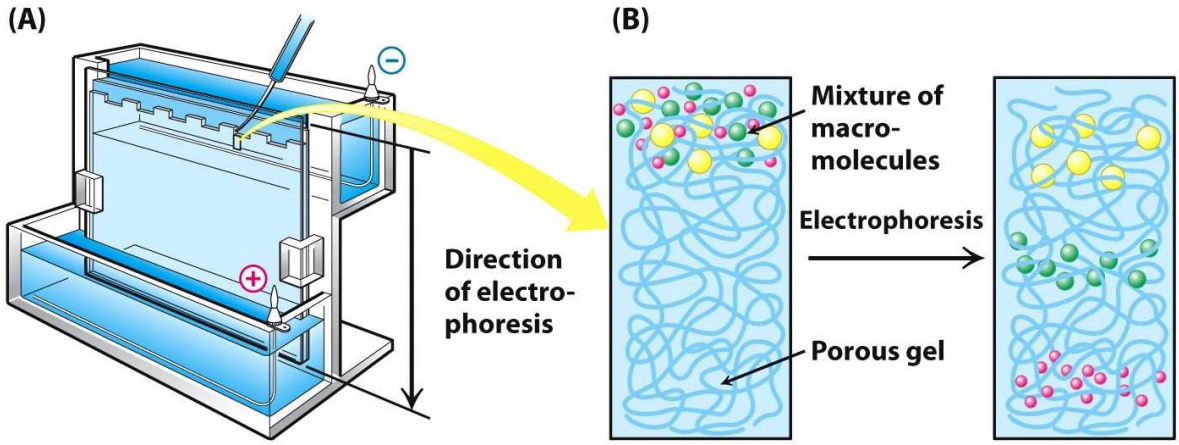

Figure 7: Vertical gel electrophoresis scheme[24]

Conductivity between the electrodes is achieved by using a buffer solution. Intensity of set in electric field depends on ratio between used voltage and length of the gel.

Mostly used electrophoresis modification of PAGE is anionic detergent sodium dodecyl sulphate (SDS) to linearize the proteins and give them a negative charge so all the molecules

moves with similar direction to anode. Low molecular weight colorants are used to enable the observation of the moving line[19; 20].

2.2.2.2 FTIR - ATR spectroscopy

Infrared spectroscopy is a spectroscopic method using absorption of infrared light for qualitative and semi quantitative characterization of chemical substances. This method is suitable for liquid, solid and even gas samples. An FTIR spectrophotometer measures data in wide spectral range at once. Thanks to Fourier transformation, time dependence of absorbance (transmittance) is changed to wavelength dependence, forming the actual spectrum.

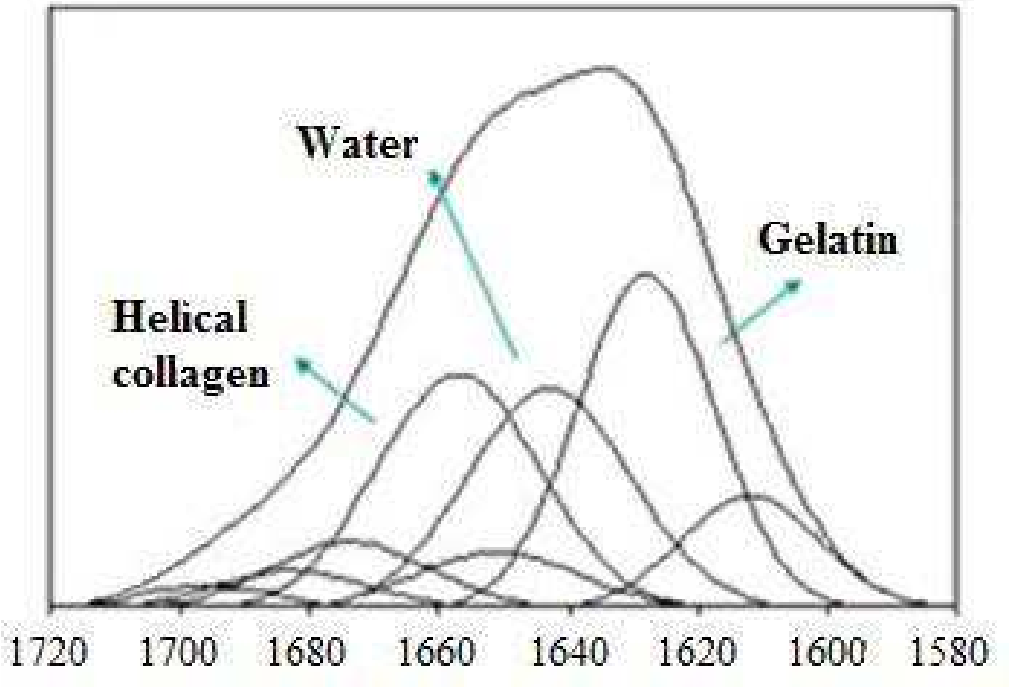

Figure 8: Displayed theoretical collagen FTIR - ATR spectrum section peak after deconvolution with shown contribution of individual components[25]

2.2.2.3 Collagen solubility

There are several ways to determine a concentration of proteins in solution. These methods usually differ in their detection limits and can be divided in five groups[20].

Table 4: Detection limits and required sample volumes for each of the methods

Determination	**Sample volume [ml]**	**Detection limit [µg.ml^{-1}]**
Biuret method	1	1 000 - 10 000
Bicinchoninic acid method	0.1	200 – 1 000
Hartree – Lowry method	1	100 - 600
Ninhydrin test	1	20 – 50
UV absorption	1	30 – 300
Bradford method	0.1 – 0.2	60 – 300
Dry matter	0.5 - 10	2 000 – 10 000

Methods based on interaction with copper

Biuret method

Method is based on interaction of copper (II) ions with peptide bonds forming a violet coordination complex in alkaline solution. This technique is suitable for sample concentration from 1 to 10 $mg.ml^{-1}$ which is 5 times diluted by the reagent to a final concentration from 0.2 to 2 $mg.ml^{-1}$. Absorbance maximum is than measured at 550 nm[13].

Bicinchoninic acid assay

This method uses a bicinchoninic acid to determine protein content and is based on alkaline protein reduction of copper (II) to a copper (I) ion and subsequent chelation of copper (I) ion by the bicinchoninic acid accompanied by a red change of colour. This technique is very sensitive to working conditions, such as temperature and time of incubation. Some substances, like ammonium ions, can have a strong interference effect and needs to be removed[20].

Hartree-Lowry method

Currently one of the most cited biochemical method in general. The reagent, first developed by Oliver H. Lowry in 1940s, has two components. First reagent is the same as for biuret method and second is Folin-Ciocalteau reagent for phenols, a mixture of phosphotungstic and phosphomolybdic acid. This technique is based on reaction of the FC reagent with copper (I) ions, produced by protein reduction of copper (I) ions. Reaction scheme also involves reduction of the FC reagent and oxidation of aromatic residues, such as tryptophan and tyrosine, accompanied by blue colour change. Hartree modified this method to increase the colour intensity and linearity among wider range of concentrations and simplify the method by using three more stable solutions. Hartree-Lowry assay is suitable for sample concentrations from 100 to 600 $\mu g.ml^{-1}$ [20].

Ninhydrin test

In this method, proteins are hydrolyzed to amino acids by 6 % sulphuric acid at 100 °C. Amino acids then react with ninhydrin and cause purple colour change. Absorbance is measured at 570 nm. Not all of the amino acids contribute to colouring in the same way. Some proteins with high amount of proline or sulphur practically do not react at all and results are misleading. Using leucine as a standard for calibration leads to satisfactory and usable results for any other protein except the one mentioned above[20].

UV absorption determination

Proteins, containing side chains of tyrosine and tryptophan, absorb light from UV area from 275 to 280 nm. If the sample is diluted enough, it is possible to determine a total protein concentration from the absorbance value in this spectrum area[20].

Bradford method

This method is based on bonding between Coomassie Brilliant blue colorant and protein molecules in acidic pH. This process is accompanied by a colour change, proportional to the protein content in sample. Bradford method is sensitive to low concentrations. This method is widely used for its simplicity and low time demand, even thou determination can be affected by interfering molecules[20].

Determination from a dry matter

In this method, proteins are dried in ovens to a constant weight at 104 or 106 °C. It usually takes a few hours and after that sample is weighted. This is the only absolute method, as it does not require any calibration or standard. Problem is high detection limitation and necessity to remove salts from the solution before drying[20].

2.2.2.4 Circular dichroism (CD) spectroscopy

A CD spectroscopy is method based on absorption of left and right circularly polarized light. As the circularly polarized light passes through the absorbing medium, its wavelength and molar absorption coefficient differ[20].

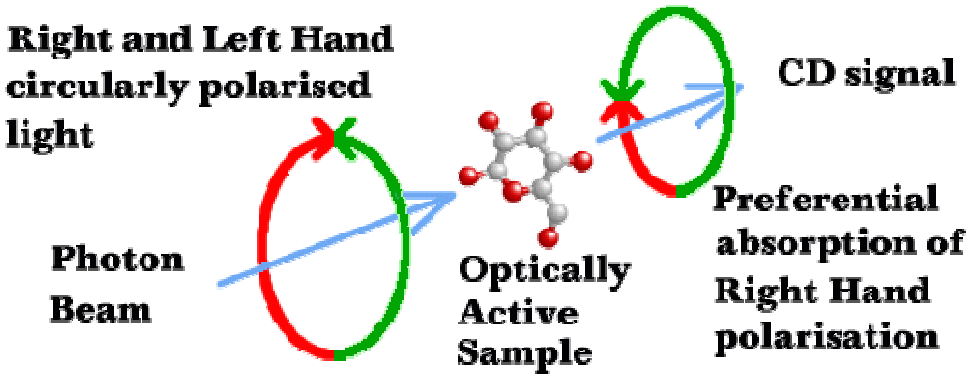

Figure 9: Principle of circular dichroism[26]

CD spectrum is dependence of the difference of molar absorption coefficients between left and right circularly polarized light on wavelength. Commonly much more often used quantity is molar ellipticity [θ], defined as:

$$tg[\theta] = \frac{OB}{OA}$$

OB ...shorter half-axis of ellipse

OA ...longer half-axis of ellipse

CD spectrum can provide information about secondary as well as tertiary structure and conformation changes of protein molecule (Fig. 10)[20].

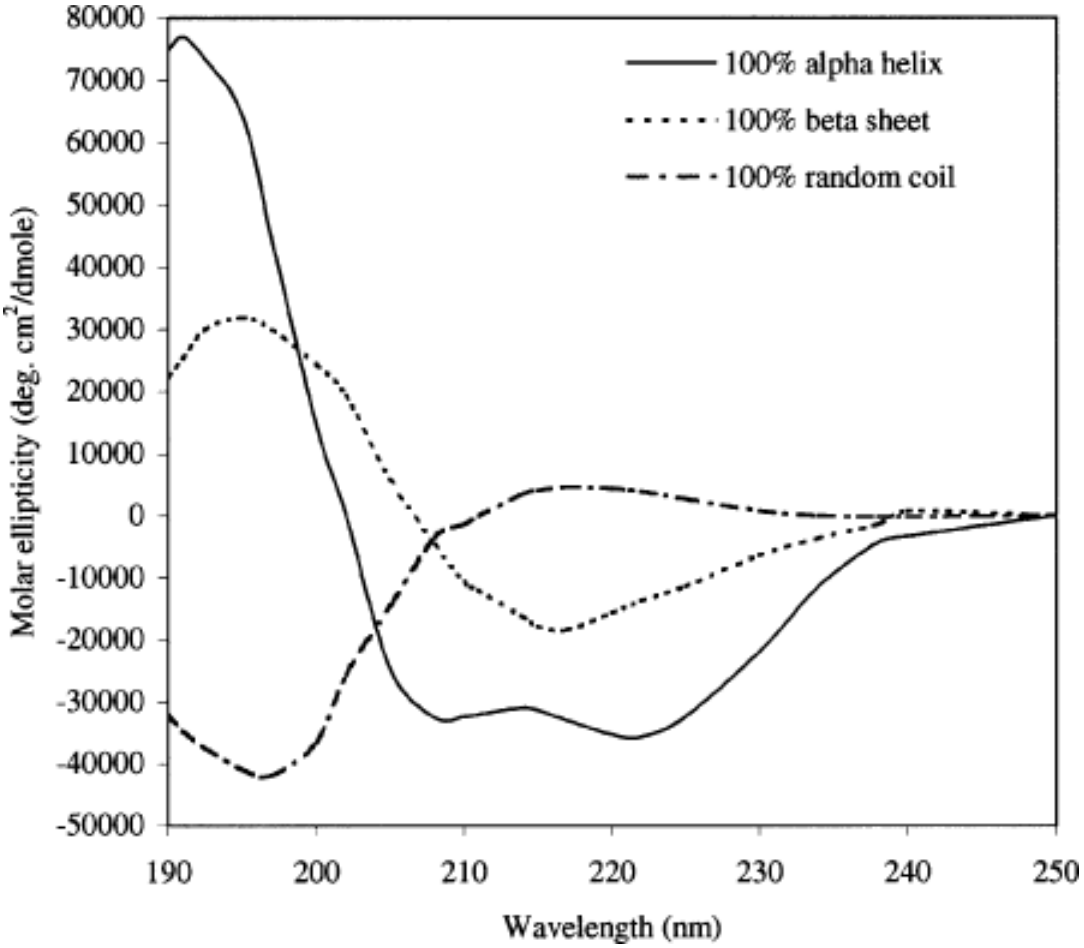

Figure 10: Typical CD spectrum of model protein conformations[27]

3 GOAL OF THE WORK

Main goal of proposed bachelor thesis is to optimize an atelocollagen separation method for purchased bovine collagen.

Specifically, to find an optimal separation conditions, such as pepsin concentration or digestion time, with a view to obtain a pure collagen at satisfactory yield. Another important part is to characterize gained product purity, stability and solubility using suitable methods.

Finally, objectively evaluate measured data and give reasons for potential mistakes and usability of used methods.

4 EXPERIMENTAL PART

4.1 Chemicals

All chemicals were used without further purification or modifications.

- Acetic acid (99.8 %, p.a.) was purchased from Lach-ner s. r. o. (Czech Republic)
- Acrylamide (AA, 40 %, p.a.) was purchased from Sigma-Aldrich (Germany)
- Ammonium persulphate (APS, 98 %, p.a.) was purchased from Sigma-Aldrich (Germany)
- Bovine collagen (8%) was purchased from VUP Medical a. s. (Czech Republic)
- Bromphenol blue (p.a.) was purchased from Sigma-Aldrich (Germany)
- Comassie brilliant blue (p.a.) was purchased from Bio-Rad (Czech Republic)
- Copper sulphate pentahydrate (98 %, p.a.) was purchased from Sigma-Aldrich (Germany)
- Ethanol was gained from school stock at Faculty of Chemistry, Brno University of Technology (Czech Republic)
- Folin – Ciocalteau phenol reagent (p.a.) was purchased from Sigma-Aldrich (Germany)
- Glycerol (99 %, p.a.) was purchased from Sigma-Aldrich (Germany)
- Glycine (99 %, p.a.) was purchased from Sigma-Aldrich (Germany)
- Hydrochloric acid (35 %, p.a.) was purchased from Lach-ner s. r. o. (Czech Republic)
- N,N´-methylenebisacrylamide (BIS, p.a.) was purchased from Sigma-Aldrich (Germany)
- N,N´-tetramethylenediamine (TEMED, 99 %, p.a.) was purchased from Sigma-Aldrich (Germany)
- Pepsin from porcine gastric mucosa (p.a.) was purchased from Sigma-Aldrich (Germany)
- Potassium chloride (99 %, p.a.) was purchased from Lach-ner s. r. o. (Czech Republic)
- Potassium phosphate (p.a.) was purchased from Lach-ner s. r. o. (Czech Republic)
- Sodium carbonate (99 %, p.a.) was purchased from Lach-ner s. r. o. (Czech Republic)
- Sodium dodecyl sulphate (SDS, 98.5 %, p.a.) was purchased from Sigma-Aldrich (Germany)
- Sodium hydroxide (98.5 %, p.a.) was purchased from Lach-ner s. r. o. (Czech Republic)
- Sodium chloride (p.a.) was purchased from Lach-ner s. r. o. (Czech Republic)
- Sodium phosphate (99 %, p.a.) was purchased from Lach-ner s. r. o. (Czech Republic)
- Sodium potassium tartrate tetrahydrate (99 %, p.a.) was purchased from Sigma-Aldrich (Germany)
- Soluble bovine dermis collagen (0,5%) was purchased from KOKEN CO. (Japan)
- Ultrapure water (ultrapure water Type I according to ISO 3696) was prepared on Millipore purification system (MilliQ Academic, Millipore, France)

4.2 Atelocollagen preparation

Atellocolagen was separated from commercially available bovine collagen so the extraction steps of the process were excluded.

General structure of process was taken over from literature, yet some modifications were applied according to measured data during the work. Figure 11 shows model separation scheme of process ideal development.

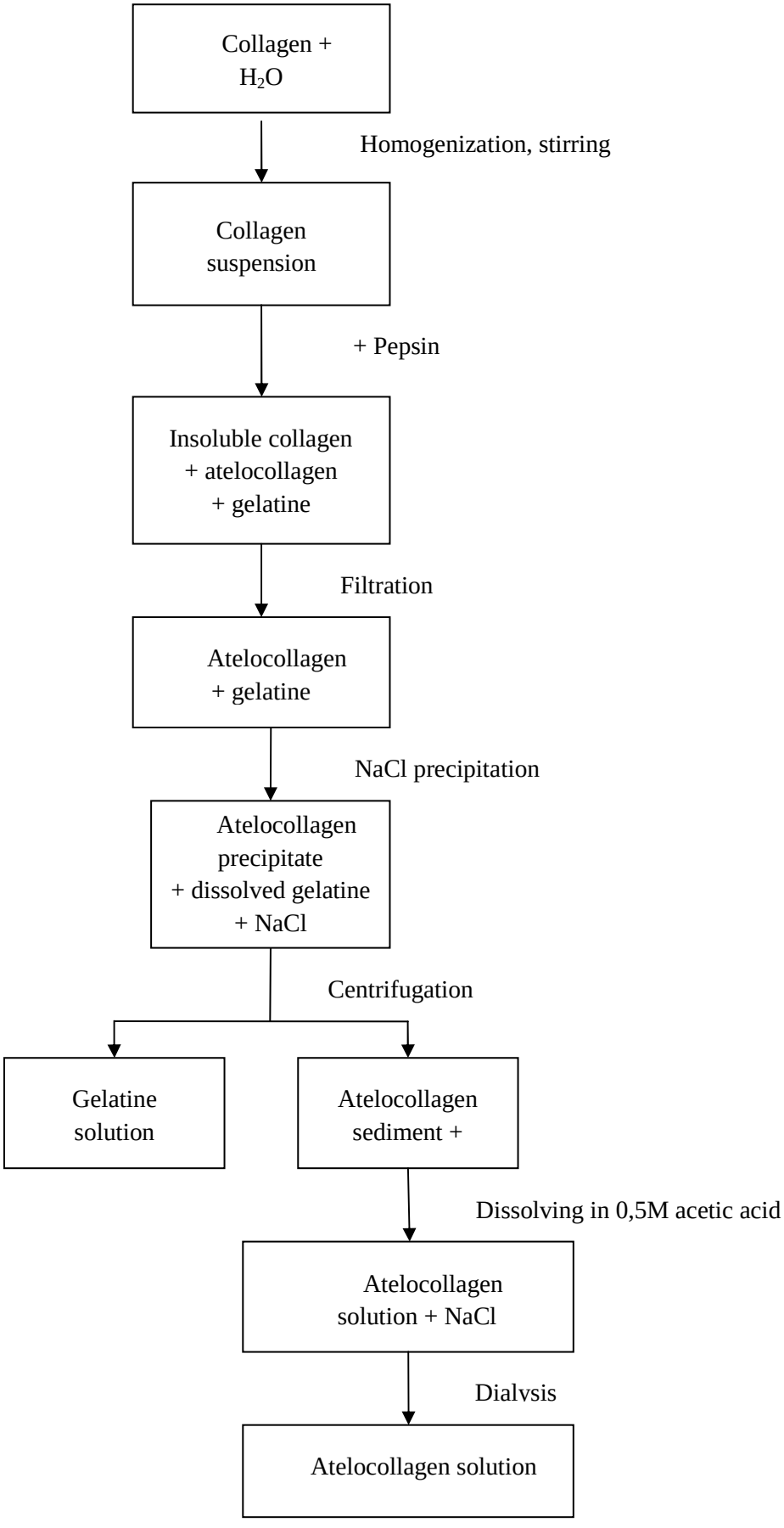

Figure 11: Atelocollagen model separation scheme

4.2.1 Preparation of collagen sample

Before digestion, every sample was cut into small pieces by scissors and water at desirable ratio was added. Suspension was homogenized by homogenizer at position 1. Mixture was then stirred for 24 hours using magnetic stirrer at various rounds per minute either at room temperature (25 ˚C) or in the fridge (4 ˚C). Used sample/water (w/v) ratio ranged from 1:20 to 1:100 and rounds per minute were either 200 or 400, at the beginning of the work not monitored at all.

4.2.2 Protease digestion

After 24 hours of stirring, desirable amount of pepsin was added to the mixture for variable time. Used pepsin/collagen (w/w) ratio ranged from 1:10 to 1:100 and duration ranged from 12 to 72 hours.

4.2.3 Purification

4.2.3.1 Filtration

After enzyme digestion, solution was filtrated to obtain a clear solution. Many types of filtration were carried out, such as glass frits (S2, S3), metal and paper filters at defined pores or ultrafiltration on membrane filters. Filtration by these methods yet proved insufficient and was replaced by centrifugation or combination of more subsequently followed filtration and centrifugation steps. Centrifugation steps differed in rotates per minute, duration and temperature. Filtration and centrifugation steps were carried out at room temperature.

4.2.3.2 Precipitation

Collagen in the filtrate was then precipitated by adding sodium chloride to a various final concentration. Used concentration ranged from 0.7 to 4 M solution. Before precipitation, enzyme activity was stopped by adding a PBS buffer solution with pH 11, modified by sodium hydroxide, to pH around 8.5. This pH modification was not used at every sample as the sodium chloride precipitation came out to be sufficient even without pH modification.

4.2.3.3 Centrifugation

Precipitate was collected using high speed centrifuge (High Speed Brushless Centrifuge MPW-350R, MPW Medical Instruments, Poland) with time, temperature and rotates per minute set as variables. Time of centrifugation ranged from 10 to 90 minutes, temperature from 4 to 20 ˚C and rotates per minute were usually set on maximum according to each rotor option possibilities (12 000 or 15 000 rounds per minute).

Supernatant was put through precipitation once or twice again according to amount of collagen that remained in solution.

4.2.3.4 Dialysis

Sediment, obtained by centrifugation, was collected and dissolved in 0.5 M acetic acid. Solution was dialysed using dialysis membrane tubes (Pur-A-Lyzer Maxi 12 000 Dialysis kit, Sigma-Aldrich, Germany) to remove salts and obtain pure collagen solution. Dialysis was

performed at 4 ˚C for 72 hours against 0.1 M acetic acid, water or both followed respectively with regular change of solution.

4.2.3.5 Lyophilization

After removing undesirable components, solution was frozen in freezer or in the freeze-dryer (Alpha 2-4 LSC Freeze-Dryer, Christ, Germany) shelf and freeze – dried to obtain dry product using a lyophilization equipment.

Prepared samples

Table 5: Prepared sample labelling with variable specifications

Sample	Temperature [°C]	Collagen/ water (w/v)	Collagen/ pepsin (w/w)	Digestion time [h]	Filtration	Precipitation	Centrifugation (4 °C)
1	Room (25 °C)	1:250	1:15	72	Frit S2	To 4 M NaCl solution	15 000 rpm, 3x 10 minutes
2		1:500					
3		1:1 000					
4	Room (25 °C)	1:200	1:25	24	Frit S2	To 2 M NaCl solution	15 000 rpm, 3x 20 minutes
5					Frit S2 + metal filter 200 µm		
6					Frit S2 + metal filter 200 µm and 25 µm		
7	4 °C	1:600	1:25	72	Frit S2	To 2 M NaCl solution	15 000 rpm, 30 minutes
8					ctfg 10 000 rpm, 30 minutes		
9					ctfg 15 000 rpm, 30 minutes		
10	Room (25 °C)	1:200	1:25	12	Frit S2	To 2 M NaCl solution	15 000 rpm, 10 minutes
11				28			
12				30			
13				33			
14				35			
15				48			
16	4 °C	1:600	1:10	72	Frit S2	To 2 M NaCl solution	15 000 rpm, 30 minutes
17					ctfg 10 000 rpm,		

					30 minutes		
18					ctfg 15 000 rpm, 30 minutes		
19	Room (25 °C)	1:200	1:25	24	Frit S2	To 2 M NaCl solution	15 000 rpm, 10 minutes
20				29			
21				32			
22	Room (25 °C)	1:200	1:25	40	Frit S2, S3, filter paper, ctfg 15 000 rpm, 90 minutes	To 0.7 M NaCl solution	15 000 rpm, 30 minutes
23				48			
24				66			
25				75			

4.3 Methods of characterization

4.3.1 PAGE – SDS electrophoresis

Polyacrylamide gel electrophoresis was performed for quality analysis of products according to their molecular weight composition. Obtained bands were then compared to specific known bands of a bovine protein standard.

4.3.1.1 Solutions

Solutions were prepared in advance. Only exception was solution E which was prepared fresh every time.

Solution A:

30 % mixture of acrylamide and bisacrylamide (8.76 g of AA and 0.24 g of BIS dissolved in 15 ml and after swelling fill up to 30 ml)

Solution B:

1,5 M TRIS-HCl buffer pH 8.8 (5.445 g of TRIS dissolved in 30 ml of distilled water, pH is modified with 6M HCl)

Solution C:

1 M TRIS-HCl buffer pH 6.8 (0.484 g of TRIS dissolved in 40 ml of distilled water, pH is modified with 6 M HCl)

Solution D:

10 % solution of SDS (1 g of SDS dissolved in 10 ml of distilled water)

Solution E:

10 % solution of ammonium persulphate (100 mg of APS dissolved in 1 ml of distilled water)

Solution G:

Mixture of 100 mM TRIS-HCl buffer pH 6.8 – 4 % SDS, 20 % glycerol, 0.02 % bromphenol blue (0.4 g SDS, 0.02 g bromphenol blue, 2 ml of glycerol, 8 ml of 1M TRIS-HCl buffer pH 6.8)

Solution F:

Electrode buffer: 0.025 M TRIS, 0.192 M glycine and 0.1% SDS (3.025 g of TRIS, 15 g of glycine and 1 g of SDS dissolved in 1000 ml of distilled water)

4.3.1.2 Gel preparation

Electrophoresis was performed at various gel concentrations. Resolving gel was used at concentrations range from 6 % to 10 % according to desired molar weight scale sharpness. Stacking gel was used at concentration range from 3 to 5 %.

Table 6: Solution content in prepared resolving and stacking gel

	10 % resolving gel	**x % resolving gel**	**5 % stacking gel**
Distilled water	2 ml	x_1 ml	1.4 ml
Solution A	1.65 ml	x_2 ml	0.330 ml
Solution B	1.25 ml	1.25 ml	-
Solution C	-	-	0.250 ml
Solution D	0.05 ml	0.05 ml	0.02 ml
Solution E	0.05 ml	0.05 ml	0.02 ml
TEMED*	0.002 ml	0.002 ml	0.002 ml
Total volume	5.002 ml	5.002 ml	2.022 ml

*TEMED = N,N´-tetramethylenediamine

Resolving gel preparation

At first, apparatus for gel electrophoresis (2-Gel Tetra and Blotting Module Electrophoresis station, Bio-Rad, Czech Republic) was assembled. Resolving gel was then prepared in desired concentration according to Table 5. Amount of distilled water and solution A were calculated as follows:

$$x = 6\ \% = 0.06$$

$$V = 5.0\ ml$$

$$x_A = 30\ \% = 0.3$$

$$x_{A+H_2O} = \frac{2+1.65}{5.0} = 0.73$$

$$x_1 = \frac{x \cdot V}{x_A} = \frac{0.06 * 5.0}{0.3} = 1\ ml$$

$$x_2 = x_{A+H_2O} \cdot V - x_1 = 0.73 \cdot 5.0 - 1 = 2.65\ ml\,.$$

Where x stands for desired gel concentration, V for final volume, x_A for solution A concentration, x_{A+H2O} for concentration of solution A and water in final volume, x_1 for amount of solution A and x_2 for amount of water.

At the earliest point, water was mixed with solution A, B, D and TEMED. Mixture was then degassed in ultrasonic bath for five minutes. Finally, solution E was added and mixture was carefully stirred. Solution was then applied between the glassware in the apparatus and immediately covered with distilled water. Solidification of gel usually took about 1 hour. Afterwards, covering water above the gel was removed by suction with filter paper.

Stacking gel preparation

Amount of distilled water and solution A for desired stacking gel concentration was calculated in the similarly as for resolving gel:

$$x = 3\ \% = 0.03$$

$$V = 2.0\ ml$$

$$x_A = 30\ \% = 0.3$$

$$x_{A+H_2O} = \frac{1.4 + 0.33}{2.0} = 0.86$$

$$x_1 = \frac{x \cdot V}{x_A} = \frac{0.03 * 2.0}{0.3} = 0.2\ ml$$

$$x_2 = x_{A+H_2O} \cdot V - x_1 = 0.86 \cdot 2 - 0.2 = 1.52\ ml\ .$$

Where x stands for desired gel concentration, V for final volume, x_A for solution A concentration, x_{A+H2O} for concentration of solution A and water in final volume, x_1 for amount of solution A and x_2 for amount of water.

First, water was mixed with solution A, C, D and TEMED and degassed in ultrasonic bath for five minutes. Finally solution E was added and whole mixture was gently stirred. Solution was applied on solid resolving gel and comb was immersed to form a sample wells in it. Solidification of stacking gel took approximately 30 minutes. Afterwards, comb was carefully removed and gel was ready to use.

4.3.1.3 Sample preparation

Sample concentration for electrophoresis was set at 3 $mg.ml^{-1}$ 0.1 M acetic acid. Samples were mixed with sample buffer at sample/sample buffer (v/v) ratio 1:1. Mixture was then incubated in water bath at 100 °C for 2 minutes. Used sample volume at each gel was 20 µl.

4.3.1.4 Electrophoresis execution

After samples were dosed to the gel wells, electrophoresis source (Power Pac Basic source, Bio-Rad, Czech Republic) was turned on. Used voltage ranged from 170 to 250 V and current ranged from 10 to 30 mA. Electrophoresis usually took from 4 to 6 hours according to the gel length and thickness. Electrophoresis was stopped when the moving blue line signifying the sample position was about 3 cm above the gel bottom.

4.3.1.5 Gel staining

After electrophoresis, gel was separated from the glass apparatus and washed with distilled water. Washed gel was then incubated in gel colorant for about 30 minutes until the bends were clearly visible. Coloured gel was washed in distilled water to remove excess colour from the gel apart from the protein bends.

4.3.2 Hartree – Lowry method

Out of many options, Hartree – Lowry method was chosen to determine collagen solubility in solution for its simplicity, reactant stability and wide use among studied literature.

At first, solutions that are necessary for determination were prepared.

4.3.2.1 Solutions

Solution A and B can be prepared in advance. Solution C needs to be prepared fresh every time. Solution B needs to be stored in fridge, solutions A is stable enough to be stored at a room temperature.

Solution A:

Sodium potassium tartrate tetrahydrate – 2 g, sodium carbonate – 100 g, 1 M sodium hydroxide solution – 500 ml, fill up to 1 000 ml with distilled water

Solution B:

Sodium potassium tartrate tetrahydrate – 2 g, copper sulphate pentahydrate – 1 g, 1 M sodium hydroxide solution – 10 ml, distilled water – 90 ml

Solution C:

Folin – Ciocalteau reagent/ distilled water at ratio 1:15

4.3.2.2 Collagen concentration in solution determination

To a 1 ml of collagen sample was added 0.9 ml of solution A and solution was incubated in 50 °C water bath for 10 minutes. Then, the solution was cooled to a room temperature and 0.1 ml of solution B was added and solution was incubated at the room temperature for 10 minutes. Finally, 3 ml of solution C were added and final solution was again incubated in 50 °C water bath for another 10 minutes.

Absorbance of calibration line samples and determinate collagen samples was measured in 1cm cuvette against blank sample at 650 nm.

4.3.3 FTIR – ATR spectrometry

Infrared spectra of gained products were measured to investigate the ratio between collagen and gelatine amount in the sample by comparing shape of absorbance peaks contribution for these two components.

Spectra were measured on Bruker Tensor 27 FTIR (Fourier-transform infra-red) spectrophotometer with ATR (attenuated total reflection) attachment with diamond crystal.

Measurements were carried out in absorbance mode at 32 scans per minute. Spectrum part of interest ranged from 1580 to 1720 cm^{-1}. Samples were measured in dry solid state.

Before every sample, it was necessary to measure the background. After that, small amount of sample was clamped by a crystal to an underlay of the ATR attachment and cover of the measure chamber was shut. Chamber was left to stabilize for about two minutes and then the sample was measured.

5 RESULTS AND DISCUSION

5.1 Separation process

5.1.1 Yields of separated products

At the beginning of the work, yields of prepared collagen samples were mostly inconsiderable (Figure 12, samples 1-3 and 7-10) and even if there were some decent yields (Figure 12, samples 4-6), they were either caused by insufficient filtration of insoluble component or visibly contaminated. It means that even high yields are not sufficient if they are not accompanied by purity and solubility of the product. On the picture below, there is a bar chart showing yields of all prepared samples.

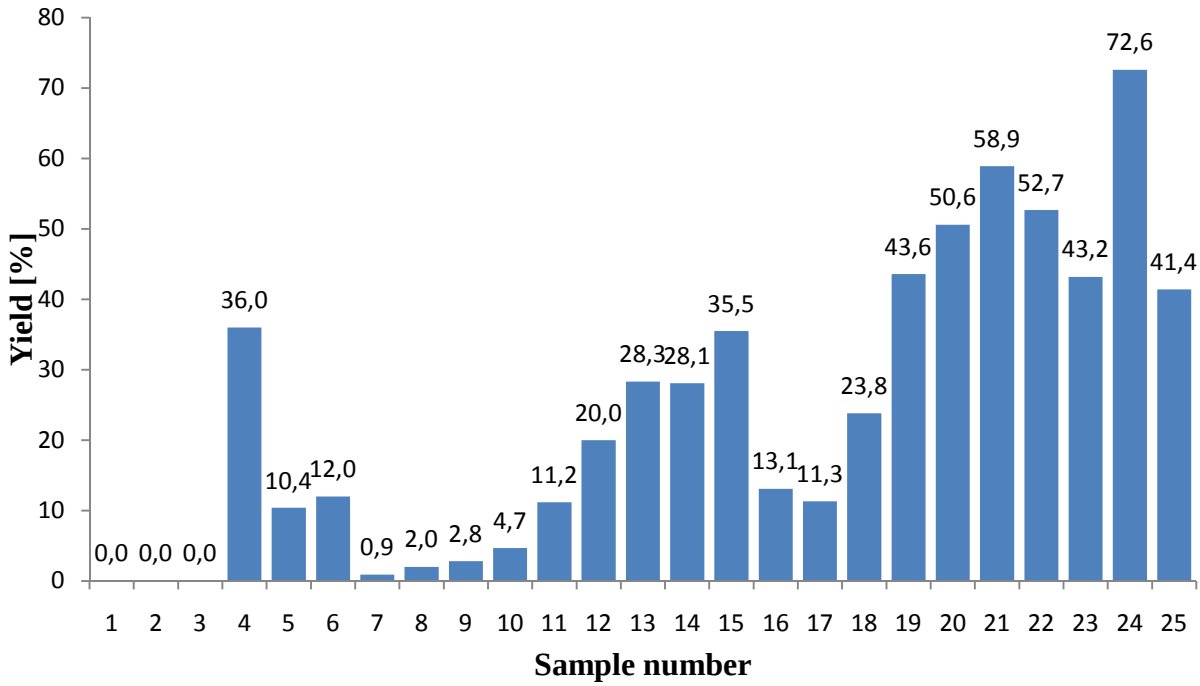

Figure 12: Yields of prepared collagen samples

As mentioned before, at the beginning of the work yields were not quite satisfactory, yet they started to improve after better familiarization with the process and each of it steps. Decent yields at the end of the work ranged from 40 % to about 60 % (Figure 12).

Factors which influenced the yield were mainly sufficient digestion time, sufficient precipitation, centrifugation and quantitative operating during the manual parts of the process. Example of yield dependence on time is shown below (Figure 13).

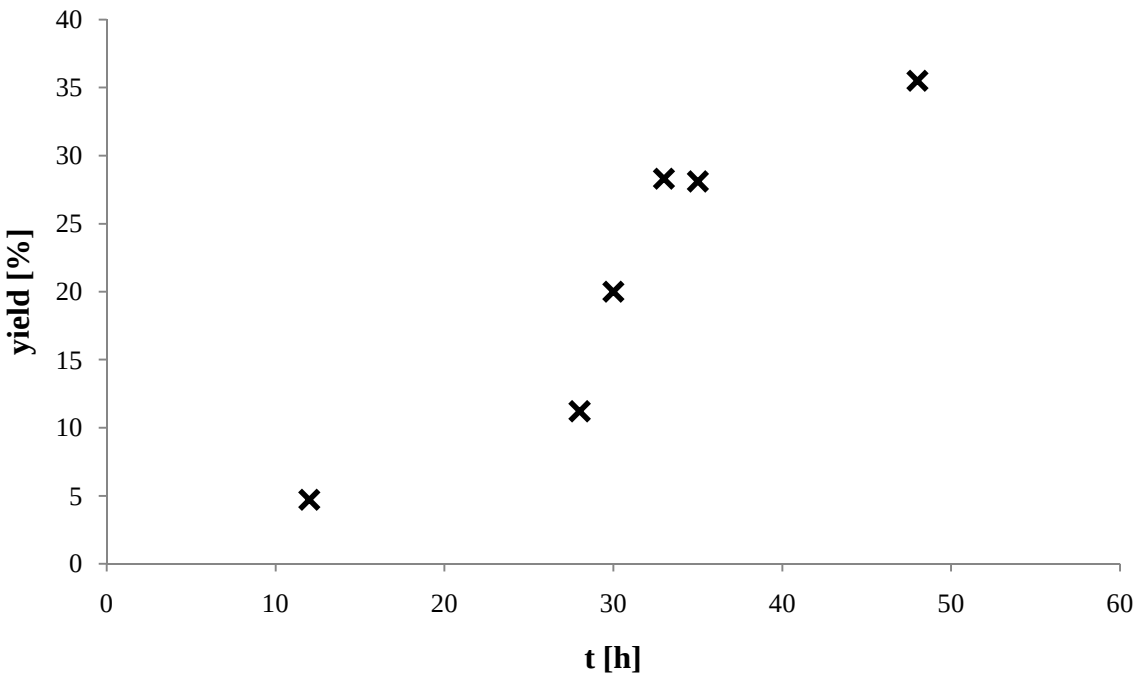

Figure 13: Example of a dependence of yield on digestion time (experiment 4)

Following graph (Figure 14) also shows reaction time dependence of a product yield. Yield 1 represents amount of collagen that was precipitated from filtrated solution and collected by centrifugation. Yield 2 represents collagen that was precipitated from the supernatant left after centrifugation of yield 1. Combined yield is simply summarization of these two yields. Growing character of the total yield on time is apparent.

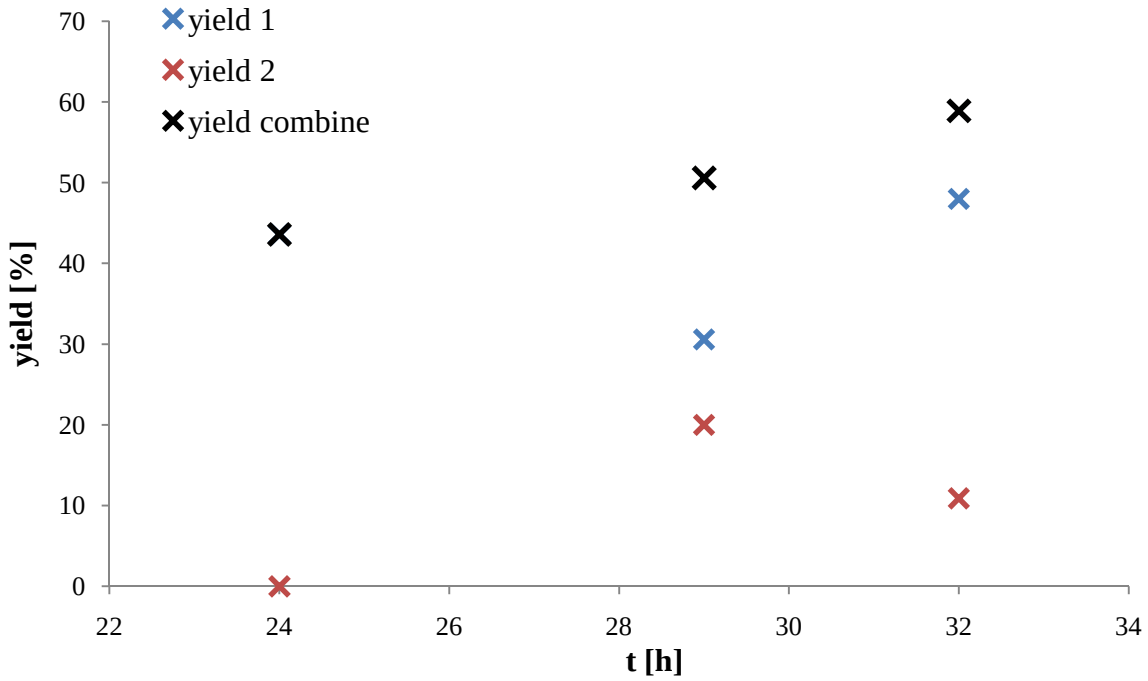

Figure 14: Dependence of collagen yield on reaction time for the first centrifugation (yield 1), second centrifugation (yield 2) and both yields combine (experiment 6)

Precipitation by NaCl confirmed sufficient at concentration 0.6 M (Figure 15). Used minimum NaCl concentration was, however, at least 0.7 M to be sure that most of the collagen is precipitated.

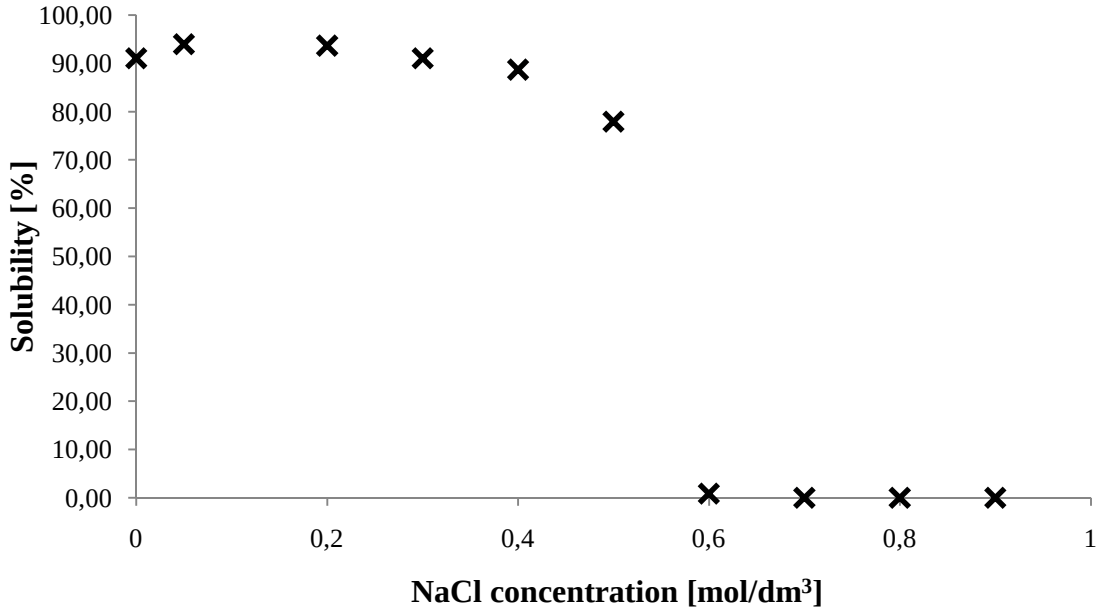

Figure 15: Dependence of reference collagen solubility on NaCl concentration in solution

Solubility, in dependence on pH, reached maximum at pH = 3 (Figure 16). pH of the prepared samples was not modified as the commercial collagen, mixed with water, had pH = 3.1.

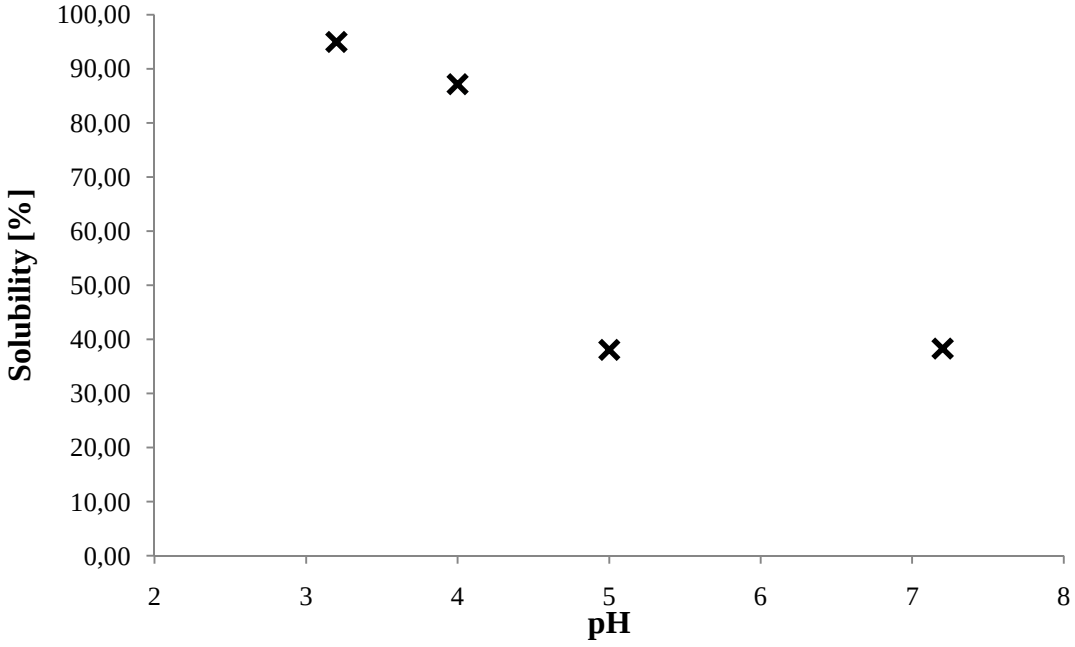

Figure 16: Dependence of reference collagen solubility on pH (pH was set using sodium acetate buffer - adding 1 M NaOH to 0.1 M acetic acid collagen solution)

5.1.2 Product purity

Purity of prepared collagen samples was mainly influenced by quality of filtration and adequate removing of salts at dialysis.

Required output of filtration step was clear solution with minimum turbidity (insoluble component, contamination etc.). Maximum observed turbidity was, as expected, in solution before filtration (Figure 17). Turbidity decreased with growing number of filtration steps included to the process – frit S2 and S3, filter paper and centrifugation (Figure 18).

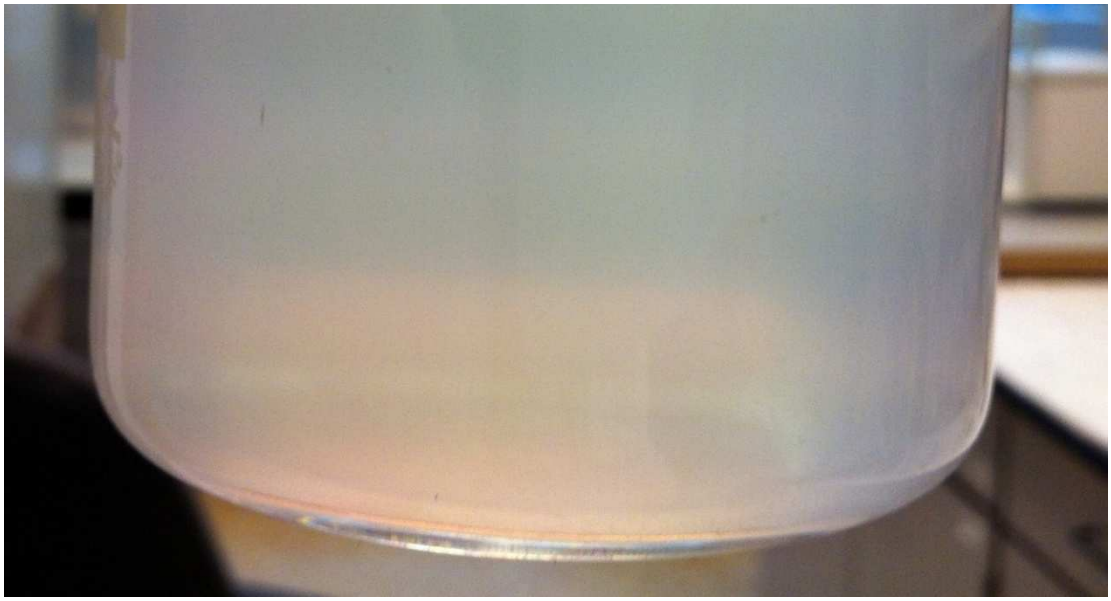

Figure 17: Solution before filtration

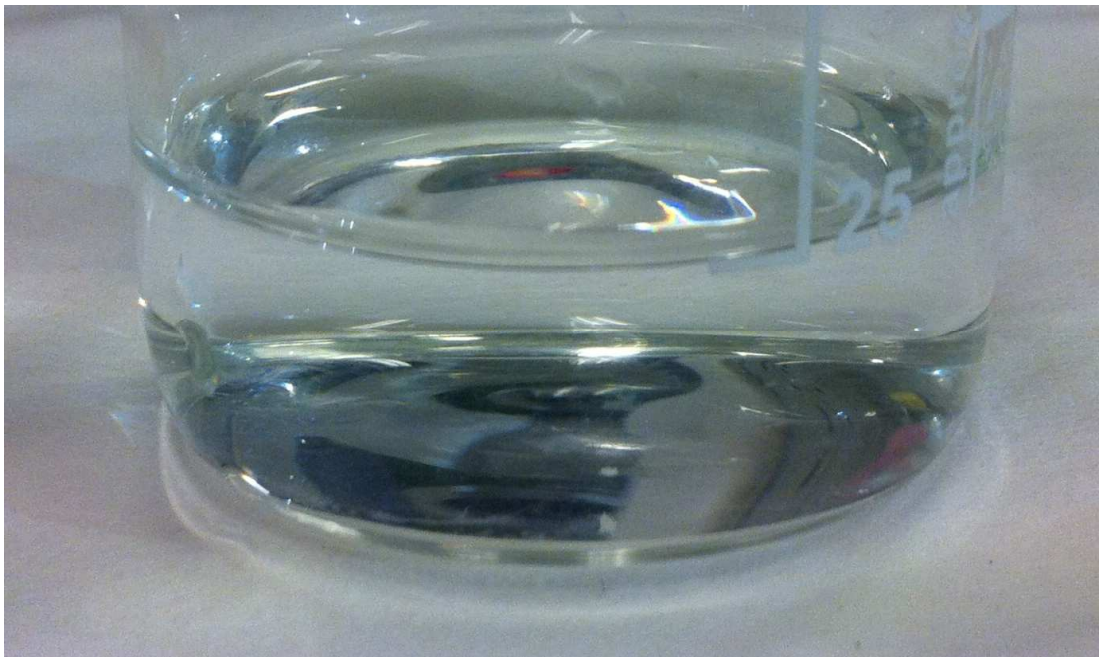

Figure 18: Solution after filtration (Frit S2, S3, filter paper and high speed centrifugation)

Dialysis lasted at least three days with periodical change of solution to maximize salt removing. Nevertheless, salt concentration in sample after dialysis was not measured. To

reassure that there is no salt left in the solution, in future measurements, silver nitrate ($AgNO_3$) could be used to form insoluble silver chloride (AgCl) and make solution turbid.

5.2 Characterization

5.2.1 PAGE-SDS electrophoresis

Polyacrylamide gel electrophoresis was used for qualitative analysis of prepared product. Condition of electrophoresis needed to bet set in effective way to enable visualization of whole range of molecular weights.

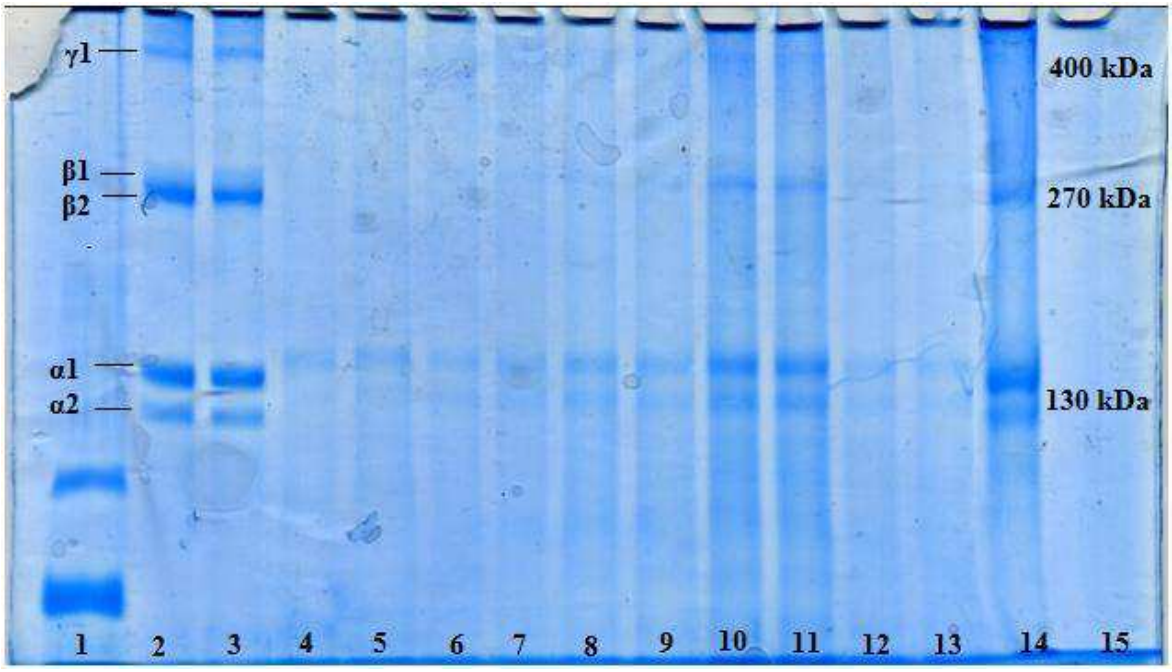

Figure 19: Example of SDS – PAGE electrophoresis of selected samples

Figure 19 shows gel electrophoresis (without using of the stacking gel) of bovine protein standard (line 1), reference material (line 2), sample 16 (lines 4 and 5), sample 17 (lines 6 and 7), sample 19 (lines 8 and 9), sample 20 (lines 10 and 11) and collagen prepared from pig skin (line 14). Picture also shows approximate positions of triple helix structural level (γ1, 400 kDa), dimer formation (β1, β2, 270 kDa) and single chains (α1, α2, 130 kDa).

Figure 20 shows gel electrophoresis of bovine protein standard (lines 1 and 2), bovine gelatine (lines 3 and 4), collagen from pig skin (lines 5-8) and source collagen purchased from VUP (lines 9 and 10). As well as previously, approximate positions of collagen structural levels along with molar weight are labelled in figure.

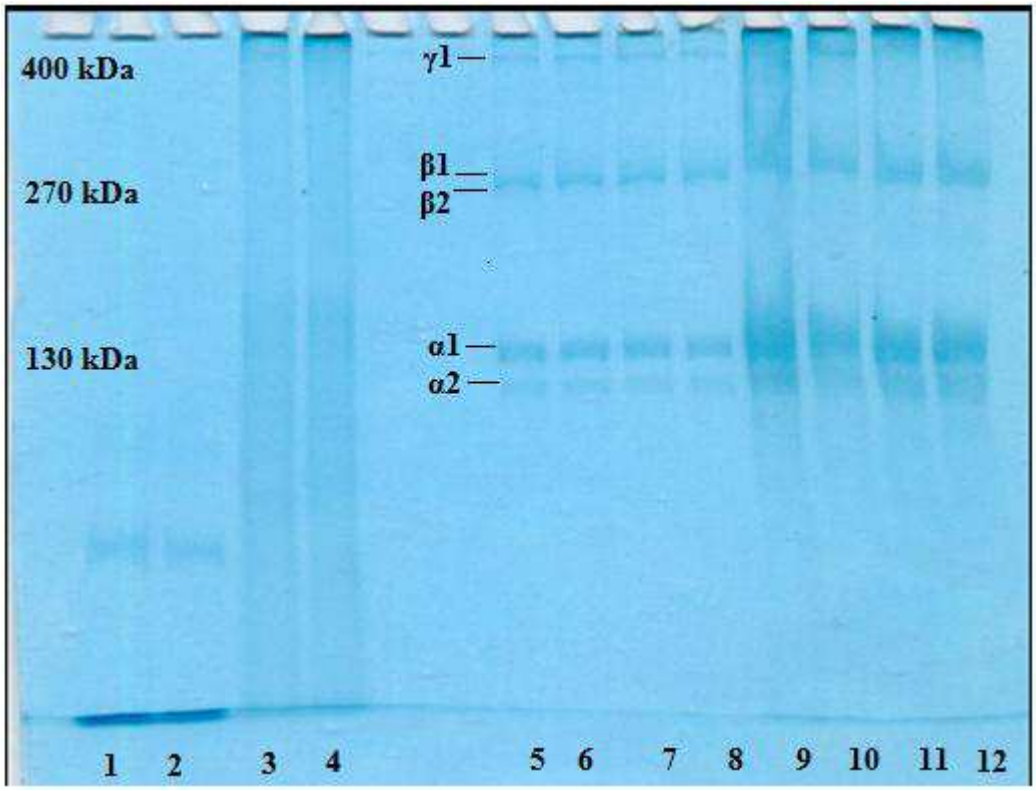

Figure 20: SDS – PAGE electrophoresis of various samples

As apparent (Figure 20), bovine gelatine sample (lines 3 and 4) does not form an accurate band at any molar mass level, but is spread all over the displayed molar mass range. Prepared collagen samples (Figure 19, lines 4-11), unlike reference sample (Figure 19, line 1-2), have visible smudgy background which is probably caused by gelatine, present in the sample. Significant amount of gelatine is also noticeable in source collagen material (Figure 20, line 9). This assumption is also obvious from FTIR-ATR spectra of source material (4.2.2, Figure 22).

5.2.2 FTIR-ATR spectrometry

Infra-red spectra were measured to study ration between helical collagen and gelatine (2.2.2.2, Figure 8) in a solid sample. Potential water content could be also visible (Figure 24).

Reference collagen spectra sequence (Figure 21) shows significant quantity of helical collagen (1660 cm^{-1}) in comparison with gelatine contribution (1630 cm^{-1}) to the resultant peak.

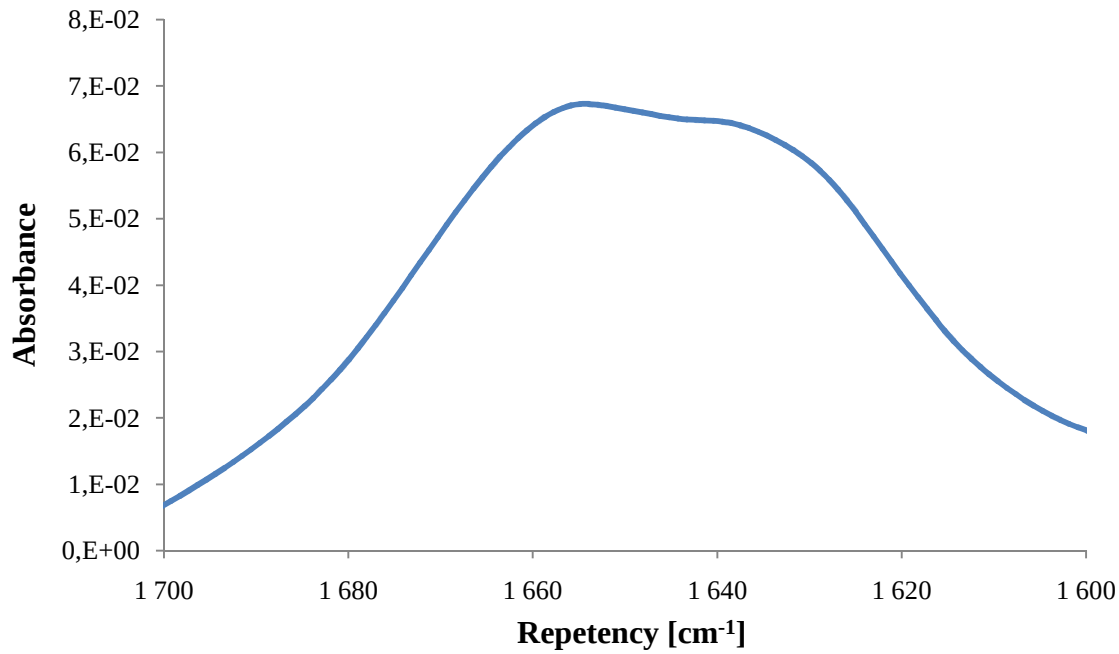

Figure 21: FTIR - ATR spectrum sequence of Koken collagen - reference material

Figure 22 shows spectra sequence of source material collagen. As mentioned before (4.2.1), substantial amount of gelatine is evident (1630 cm^{-1}).

Figure 22: FTIR - ATR spectrum sequence of VUP collagen - source material

Figure 23 shows spectra sequence of sample 16 with helical collagen contribution (1660 cm^{-1}) almost comparable with reference (Figure 21). However, significant amount of denaturated collagen is obvious (1630 cm^{-1}).

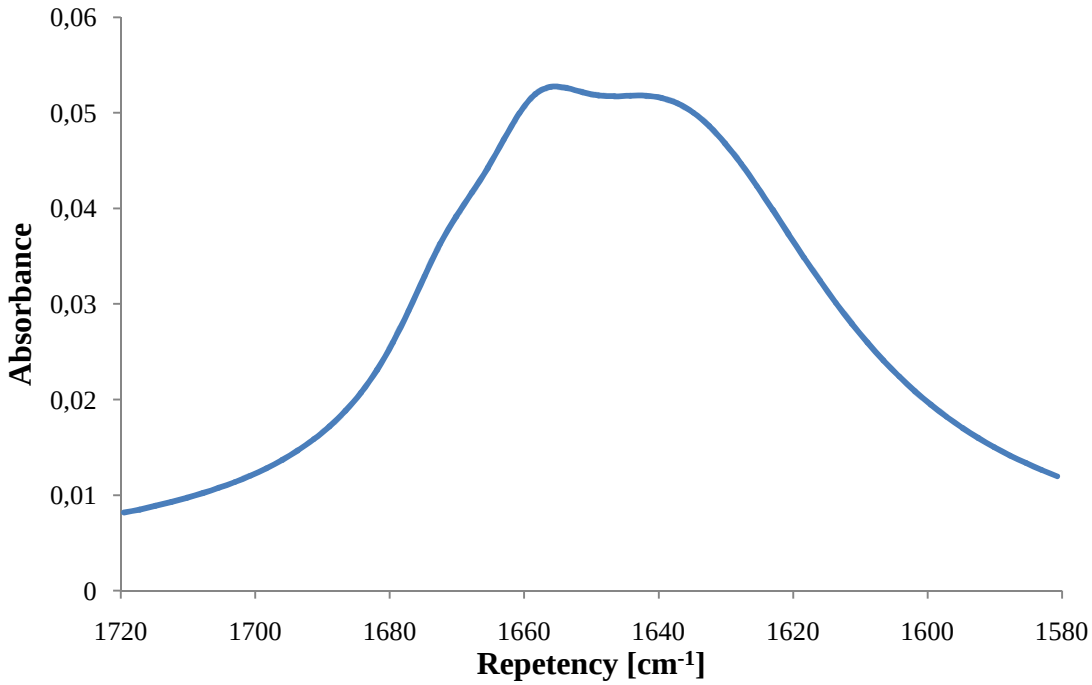

Figure 23: FTIR - ATR spectrum sequence of sample 16

Spectra sequence of sample 19 shows approximately same content of helical and denaturated collagen (Figure 24).

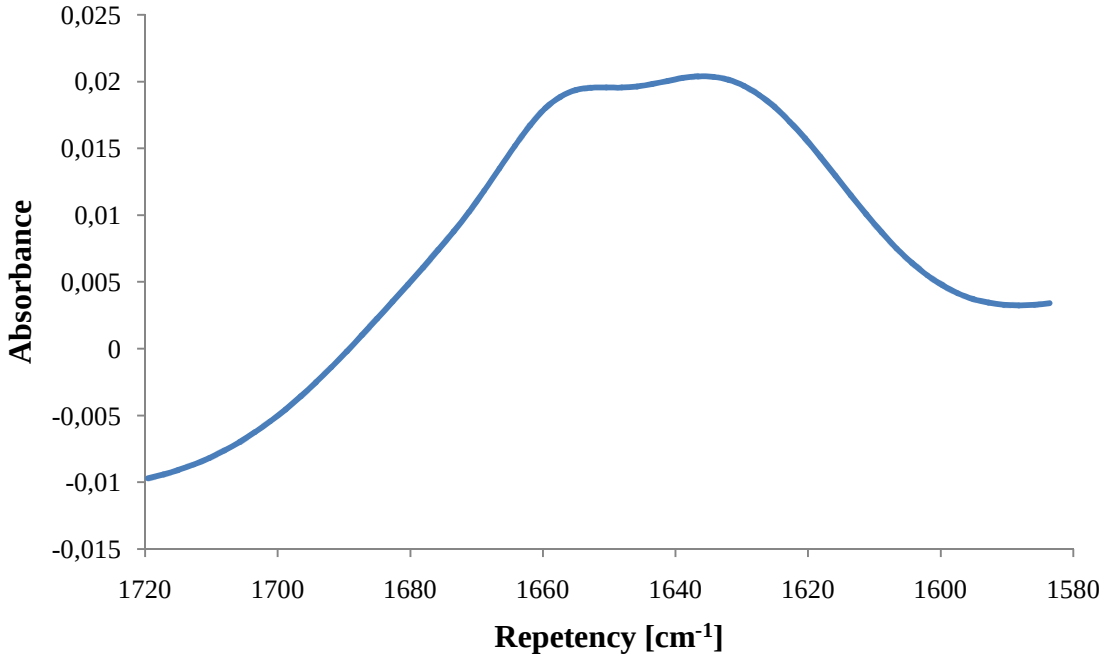

Figure 24: FTIR - ATR spectrum sequence of sample 19

Infra-red spectra of prepared samples seemed to be helpful in providing some information about the sample, however, inconsistent results were sometimes obtained as the spectra, measured on different parts of identical sample, slightly differed.

5.2.3 Collagen solubility - Hartree – Lowry method

Hartree-Lowry method turned out to be reliable in collagen solubility determination, yet only after optimizing conditions and sample preparation for this technique. Subject of optimization were mainly dilution, dosage and calibration for quantitative measurement.

Albumin standard, as used for calibration in literature, was proved unreliable. As shown in Figures 25 and 26, the molar absorption coefficient of albumin (0.003) and collagen (0.001) differs (directive in regression equation). According to that, albumin absorbance was lot higher than collagen absorbance, so using it as a standard material for calibration would lead to incorrect data.

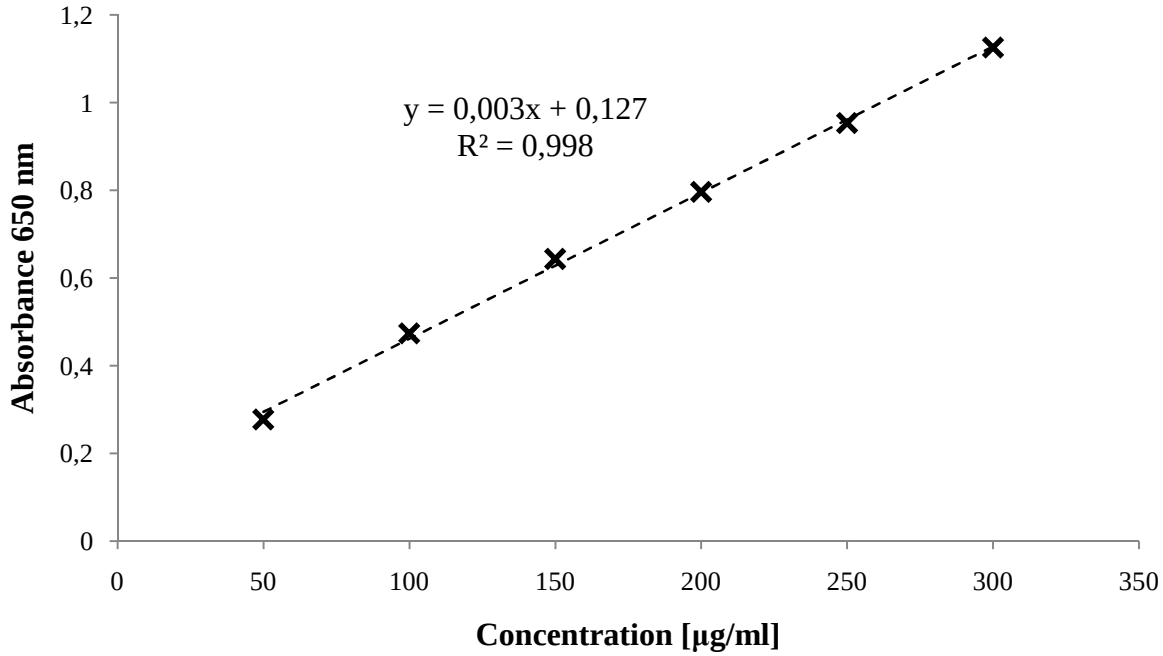

Figure 25: Calibration line of bovine serum albumin

Collagen reference material was then set as a standard for calibration line (Figure 28). Calibration dependence proved to be steadily linear, but absorbance values were usually slightly shifted. To reduce deviations, calibration line was prepared new for every measurement.

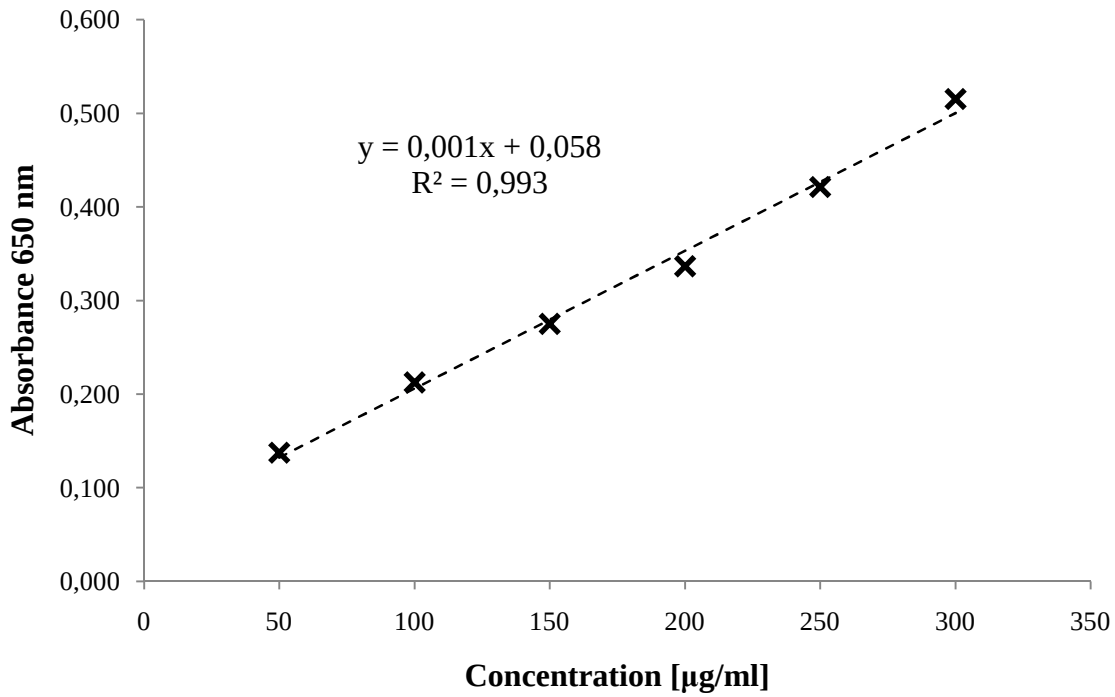

Figure 26: Calibration line of Koken collagen

Hartree-Lowry method was found useful for measuring collagen solubility dependence on NaCl concentration in solution (Figure 15) and on pH of the solution (Figure 16).

6 CONCLUSIONS

Presented work was focused on the separation of soluble atelocollagen out of insoluble tropocollagen using protease digestion and characterization of gained product quality. Process turned up to be very time consuming with wide range of variables that needed to be taken into concern. It was also complicated to discover one set of conditions that would guarantee a successful result every time. It was more likely to try various setting combinations and then supplement it with results, which can be expected.

Soluble collagen was separated out of commercially available bovine collagen, declared as type I collagen. Pepsin was chosen as an enzyme for protease digestion because of its wide use among studied literature and good availability.

Preparation of purchased collagen for pepsin digestion included cutting and homogenizing the sample with solution. Variables in this step covered sample/water (w/v) ratio and rotates per minute on magnetic stirrer. Sample/water ratio 1:200 (0.5 % solution) was found out to be convenient. Rounds per minute were set on 400.

Pepsin digestion of collagen sample was crucial for final product both quantity and quality. Pepsin/sample (w/w) ratio and digestion time were set as variables. Pepsin/sample ration was found out to be sufficient at 1:25. Time of digestion was tougher to determine. Time under 48 hour proved to be insufficient, providing inconsiderable yield and lot of insoluble material. After approximately 80 hours of digestion, solution started to turn yellow with emerging gelatine being prevalent. Optimal time for digestion, providing satisfactory yields (40-60 %), with most probability lies between those two values, most likely from 60 to 70 hours.

Filtration of solution after digestion was another important step of the process. Obtaining a clear solution was decisive mainly for product purity and follow-up solubility. Sufficient was combination of filtration by S2 and S3 frit, filter paper and subsequent centrifugation at 15 000 rpm, 20 °C for 90 minutes.

Sufficient precipitation was important for maximizing the yield. From collagen standard solubility dependence on NaCl concentration was found out, that in 0.6 M NaCl solution is almost all the collagen precipitated.

Collecting the precipitate by centrifugation showed up to be sufficient at 15 000 rpm, 4 °C, for 30 minutes. Dialysis lasted at least 3 days with regular change of solution.

Freezing of the samples was best in the shelf of the freeze-drier, where the drying took place.

Characterization of samples by gel electrophoresis served for qualitative determination of product molecular weight. Electrophoresis also partially showed if the gelatine is present in the sample (blurred background).

Hartree-Lowry method appeared to be good and relatively easy method for collagen solubility measurement, yet only with suitable calibration standard.

Infra-red spectra of prepared product provided some information about ratio between helical collagen and gelatine in solid sample. More accurate content of collagen and gelatine could be achieved by deconvolution of peaks and subsequent integration. Nevertheless, measured spectra were not fully reliable as they sometimes slightly differed within one sample. It also seemed that obtained spectra depended on some other variables and can be considered only as a rough estimate of actual structure. For further and more accurate structural analysis, circular dichroism spectroscopy could be used.

Advantageous appeared to be comparing results of mentioned characterization methods with methods that can provide similar information as it contributed to credibility of measured results.

7 LIST OF FIGURES

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9 ABBREVIATION CONTENT

AA	Acrylamide
APS	Ammonium persulphate
BIS	N,N´-methylenebisacrylamide
BSA	Bovine serum albumin
CD	Circular dichroism
Ctfg	Centrifugation
EDTA	Ethylenediaminetetraacetic acid
EDTA-2Na	Ethylenediaminetetraacetic acid disodium salt
FC	Folin-Ciocalteau reagent
FTIR	Fourier transformation infrared spectroscopy
FTIR-ATR	Fourier transformation infrared spectroscopy with attenuated total reflection
Gly	Glycine
PAGE	Polyacrylamide gel electrophoresis
PAGE-SDS	Polyacrylamide gel electrophoresis in presence of sodium dodecyl sulphate
PBS	Phosphate buffered saline
SDS	Sodium dodecyl sulphate
TEMED	N,N´-tetramethylenediamine
UV	Ultraviolet area of light
UV-VIS	Ultraviolet and visible area of light

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